A study of some factors involved in the adaptation to temperature in the freshwater crayfish austropotamobius pallipes (lereboullet)

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A STUDY OF SOME FACTORS INVOLVED IN THE ADAPTATION TO TEMPERATURE IN THE FRESHWATER CRAYFISH *Austropotamobius pallipes* (Lereboullet).

with an Appendix: *Thelohania contejeani* Microsporidian parasite of Crayfish muscle

by

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GLOSSARY

^{o}A - degrees absolute.

ATP - adenosine 5'-triphosphate.

ATPase - adenosine 5'triphosphatase (E.C.3.6.1.3.).

BHT - Butylated hydroxytoluene (antioxident).

BSA - Bovine Serum Albumin.

^{o}C - degrees Centigrade.

$[Ca^{2+}]$ - calcium concentration.

Ca^{2+} ATPase - Calcium-stimulated adenosine 5'-triphosphatase (E.C.3.6.1.3.).

Cd - Cardiolipin.

Ea - Activation Energy.

EDTA - Ethylene diamine tetraacetic acid.

EGTA - Ethylene glycol-bis(β-amino-ethyl(ether)N,N'-tetra-acetic acid.

E-S - (Enzyme-substrate).

FSR - Fragmented sarcoplasmic reticulum.

ΔG^{*} - Change in Gibbs free energy during enzyme activation.

GLC - Gas liquid chromatography.

ΔH^{*} - Change in enthalpy during enzyme activation.

h - height of chromatographic peak.

h^{1} - Planks constant.

Hepes - N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid.

^{o}K - degrees Kelvin.

k - rate constant.

K_{B} - Bottzmann's constant.

K_{M} - Michaelis constant

LD_{50} - Time for 50% mortality in a sample of animals, or time for enzyme activity to be reduced by 50%.

Mg^{2+}ATPase - Magnesium-dependent, adenosine 5'-triphosphatase (E.C. 3.6.1.3.).

$(Na^{+} + K^{+})$ATPase - Sodium and potassium stimulated, magnesium dependent adenosine 5'-triphosphatase (E.C. 3.6.1.3.).

n,N	-	number of observations.
PC	-	choline phosphoglycerides.
PE	-	ethanolamine phosphoglycerides.
PI	-	inositol phosphoglycerides.
P_i	-	inorganic phosphate.
PS	-	perine phosphoglycerides.
r	-	correlation coefficient.
K	-	Gas constant.
Rt	-	retention time.
RRt	-	relative retention time.
S	-	substrate concentration.
Sph	-	Sphingomyelin.
ΔS^*	-	change in entropy during enzyme activation.
T	-	temperature.
TLC	-	thin-layer chromatography.
Tris	-	Tris(hydroxymethyl)aminomethane.
V	-	reaction velocity.
Vmax	-	maximal reaction velocity.
W	-	peak width at base cut by tangents.
$W_{\frac{1}{2}}$	-	peak width at half-height.

CONTENTS

ABSTRACT

The phenomena of resistance compensation to temperature and heat death in the freshwater crayfish Austropotamobius pallipes has been studied, with particular regard to muscle membranes.

The lipid composition of whole muscle was analysed by conventional chromatographic techniques. The principle phospholipids present were choline phosphoglycerides (50%), ethanolamine phosphoglycerides (25%) and serine/inositol phosphoglycerides (10%). Temperature acclimation had no effect on the phospholipid composition.

The fatty acid composition of various phospholipid classes were also analysed by GLC and were found to be highly unsaturated. Total phospholipid extracts from animals acclimated to 4°C (18 hr light photoperiod) contained a slightly greater proportion of unsaturated fatty acids, compared to extracts from 25°C acclimated crayfish. The acidic phospholipids showed the greatest changes.

Acclimation to 4°C with 8 hr light photoperiod conditions caused incorporation of a still greater proportion of unsaturated fatty acids into membrane phospholipids. This daylength effect, however, was not associated with a change in the thermal resistance of the whole animal.

The overall activity of the Ca^{2+}-stimulated ATPase of crayfish sarcoplasmic reticulum was unaffected by thermal acclimation, although warm-acclimated crayfish yielded significantly more microsomal protein per gram muscle weight.

The kinetics characteristics of enzyme activity were independent of temperature up to 28°C. Incubation at 35°C (a lethal temperature) caused a decline in enzyme-substrate affinity. Thermal acclimation had no effect upon these characteristics.

Arrhenius plots of enzyme activity for preparations isolated from both acclimated groups were linear. The activation energy for the enzyme from each acclimation group was similar (approximately 15 Kcals. $mole^{-1}$; 62.8 KJ. $mole^{-1}$).

The process of inactivation of the Ca^{2+}-stimulated ATPase at lethal temperature was also studied. High KCl media protected the enzyme from inactivation, although dilution, and addition of BSA and calcium had no effect. Inactivation in low KCl media, was a first order process. Acclimation had no significant effect upon the rate of inactivation.

The proposed role of membranes in the phenomenon of resistance compensation has been discussed in the light of this and other data.

An ultrastructural and histological study of a Microsporidian parasite of crayfish muscle is also presented.

Chapter 1

CONCEPTS OF PHYSIOLOGICAL ADAPTATION

GENERAL INTRODUCTION

Prosser (1964) has defined physiological adaptation as "any property of an organism which favours survival in a specific environment, particularly a stressful one". Such adaptations to the environment may be the result either of physiological changes within the lifetime of an organism, or genetic selection within a species or subspecies over a large number of generations. The former phenomenon is caused by the differential expression of a single genotype in response to environmental stimuli, and has been termed "phenotypic adaptation" by Bligh and Johnson (1973). However, Fry (1967) and others have reserved the term 'adaptation' for the phylogenetic adjustments of an organism to its environment. The term 'physiological compensation' avoids confusing ambiguities with evolutionary adaptation and other biological phenomena (Hoar, 1967).

The terms acclimation and acclimatization both relate to compensatory adjustments of an organism to the climatic components of the total environment (Bligh and Johnson, 1973). Although the two words are etymologically indistinguishable, they have been assigned several different meanings and are frequently used synonomously (see Bligh and Johnson, 1973).

The most widely used meanings (Fry, 1967; Prosser, 1964; Precht, Christophersen, Hensel and Larcher, 1973), and those adopted here, use acclimation to describe physiological compensations in response to changes in one particular environmental parameter, as may be achieved within the laboratory. On the other hand, acclimatization refers to the compensatory adjustments of

an organism to the more complex changes in several environmental parameters, such as occur during seasonal changes in the natural environment. This distinction is justified in view of the major physiological differences between an organism acclimated in a laboratory to low temperatures, and one acclimatized under natural conditions to winter temperatures (Hoar, 1955; Hoar and Robertson 1959; see also Chapter 3).

Perhaps the most significant aspect of acclimatization as opposed to acclimation, is that the former process permits the organism to anticipate changes in environmental conditions, such as occur seasonally, and thus perhaps to adapt to them before they are manifest (Hoar 1967). In this respect, photoperiod represents the most reliable trigger for this 'preadaptation' phenomenon.

Temperature is probably the most pervasive and important environmental influence, particularly for temperature-conforming organisms. Its ease of measurement and control in the laboratory have made this perhaps the most useful environmental parameter for the study of compensatory phenomena. Precht (1958) has distinguished between (a) compensations that allow an organism to withstand thermal extremes, which ultimately prove lethal, and (b) compensations of metabolic activity measured over a range of tolerable temperatures. The latter phenomenon is called 'capacity' compensation (Precht 1949; translated from the German 'Leistungadaptation'), a term which originates from observations on poikilothermic organisms whose locomotory and metabolic activities increase in rate with a rise in temperature according to the Van't Hoff-Arrhenius laws. After a period of time, however, there may be a change in the rate of the process, which may to some degree compensate for the rate effect of the shift in environmental temperature. The cellular and molecular basis of these capacity compensations are currently the subject of intense

investigations (for reviews see Hochachka and Somero, 1973; Prosser, 1973; Precht, Christophersen, Hensel and Larcher, 1973; Wieser, 1973).

Precht, Christophersen and Hensel (1955) have described five different patterns of capacity compensation, which are most easily understood by reference to Figure 1-1. An animal which is acclimated at T°C yields a rate/temperature curve that rises with increasing temperature from A at T°C to B at T+10°C on Figure 1-1. If after adaptation to T+10°C, the rate for that process is restored to its original level (C) then the compensation is described as perfect or 'ideal' (Type 2 compensation) and yields a new R/T curve, C to D. If the rate is lower than the original level then the compensation is 'supraoptimal' (Type 2) and if the rate lies between points B and C, the compensation is described as 'partial', a type 3. If there is no change in the rate after a period of acclimation or acclimatization then no compensation has occurred (Precht Type 4), whilst if the rate is higher after acclimation the compensation is described as 'inverse' or 'paradoxical' (Precht Type 5).

An alternative classification has been suggested by Prosser (1973) who uses the changes in the R/T curve to characterise the capacity compensation. When no adaptation occurs after a period of acclimation, the rate/temperature curves for the differently treated animals coincide (Pattern I). Frequently there is movement of the R/T curve along the temperature axis without a change in slope (Pattern II, 'Translation') or there may be rotation of the R/T curve about a midpoint ('Rotation', Pattern III This latter phenomenon is caused by a change in the slope of the R/T curve. Commonly, the process of adaptation involves a combination of Patterns II and III, and in each case may be compensatory or non-compensatory. Each classification scheme may be used for comparing differently treated animals, their

tissues, cells, isolated subcellular fractions or enzymes, although the results for the latter systems may differ from measurements on intact animals. In general, the tolerance limits of isolated parts are greater than those in intact animals (Prosser, 1967).

When the environmental conditions exceed the normal variations tolerated by an organism, the animal is placed under stress. The organism resists the disruptive effects of the stress for a period of time that depends upon the intensity of the stress and its inherent resistance, but eventually succumbs. The physiological characteristics of an organism that permits survival or increased resistance at environmental extremes are termed resistance compensations (from the German 'Resistanzadaptation'; Precht, Christophersen and Hensel, 1955). They indicate the tolerance limits of the organism for its growth, development and reproductive processes. These tolerance limits may depend upon a variety of factors. For example, genotype (Wood, 1957; Bowler and Hollingsworth, 1965) age (Hollingsworth and Bowler 1966; White 1953; Davison 1969) previous thermal history (Bowler 1963a), and ionic acclimation (Alabaster 1967; Garside and Jordan, 1968).

An animal acclimated at a higher temperature usually becomes more tolerant of high temperatures and more resistant to lethal high temperatures. Precht, in Precht, Christophersen, Hensel and Larcher (1973) has suggested that this case should be termed 'reasonable' resistance compensations, whereas the opposite case of a decreased heat resistance with an increase in environmental temperature should be described as 'paradoxical'. Reasonable resistance compensation to both thermal extremes have been described for ciliates (Vogel, 1967), decapods (Kinne, 1964; McLeese and Wilder, 1958; Bowler, 1963a), fishes (Fry, 1971) and others (see Prosser, 1973). Paradoxical resistance compensation is reported less often. Edwards (1958) found reasonable compensation to cold in imagos of Tribolium confusum, but

paradoxical compensation in adults. According to Baldwin (1954), the parasitic hymenopteran Dahlbominus fuscipennis shows reasonable resistance compensation to higher temperatures, but paradoxical resistance compensation at lower temperatures. This was correlated with optimal formation of 'protective substances' at certain temperatures. An alternative explanation is provided by the studies of Hollingsworth and Bowler (1966), Bowler (1967) and Davison (1969). These workers have found a progressive decrease in the resistance to high lethal temperatures with time after eclosion, in Drosophila sp, Tenebrio sp, and Calliphora sp. respectively. However, the rate of decline of thermal resistance is heavily dependent upon the environmental temperature of the adult stage, in a paradoxical fashion. Adults maintained at high temperature show a more rapid decline in thermal resistance than adults maintained at low temperatures (Davison, 1969). It has been suggested by these workers that the decline in thermal resistance is caused by a loss of highly resistant pupal factors, a loss that occurs more rapidly at higher temperatures.

The precise relationship between resistance and capacity compensation is not clear, and most workers study them separately. Precht (1958) has suggested that the coupling of resistance and capacity adaptation is not obligatory since there are animals which show compensation of oxygen consumption, but do not show resistance compensation (I. Precht, 1967).

However, whilst it is possible to envisage the adaptive value of resistance compensations in situations where an organism frequently experiences lethal stresses, its value to animals that rarely encounter such conditions is difficult to see. In this latter situation there is no opportunity for these compensations to be expressed and to be favoured by natural selection. The altered resistance displayed by an organism after a period of thermal acclimcation or acclimatization may, therefore, be a

consequence or manifestation of a capacity compensation, rather than a compensation towards altered resistance *per se*. Thus, although in a few cases such physiological compensations may have ecological significance, such as at the limits of a species geographical range (for example, Reite, Maloiy and Aasehaug, 1974), it is nevertheless a reflection of compensatory adjustments. Clearly, a study of the interrelationship of these two phenomena in the same animal would clarify the situation.

A basic strategy in the investigation of compensatory phenomena has been the identification of the physiological and biochemical basis for the response. With resistance compensations it is necessary to identify the causes of death during lethal stress, since these same factors must also be modified during the compensatory process.

The factors that lead to heat and cold death in poikilotherms are not well understood at present, but in the higher organisms at least, a great many factors are probably involved. Different processes may be involved in different species or in the same species exposed to different lethal temperatures, and it is unlikely that sweeping generalisations can be made. An additional complicating factor is the observation that the kinetics and causes of heat death may be different for the intact animal, its organs, tissues, subcellular fractions or enzymes. Orr (1955) has shown that the temperatures of heat death for a 15 minute exposure in *Rana pipiens* were: intact tadpole, 37.5°C; whole frog, 38.6°C; gastrocnemius muscle, 40.2°C; heart *in situ*, 42.0°C; sciatic nerve, 43°C. Bowler (1963b) in *Austropotamobius pallipes*, and Grainger (1969) in *Arianta arboruntum* have observed vigorous oxygen uptake for up to 1 hour in tissues, at temperatures which would rapidly prove lethal for the intact organism. In general, the tolerance limits are narrowest for intact organisms, but somewhat wider for tissues and cells (Prosser, 1973).

There have been a variety of theories concerning the causes of heat death, of which two are currently widely held, the denaturation of protein and the 'melting' of lipids. Perhaps the most obvious explanation is that of protein denaturation, in the same manner that egg albumen becomes coagulated when heated in a test tube. Its most prominent advocate at present is Ushakov (1964, 1966) who was much impressed by the similarity among the temperature coefficients of the heat death of cells, of protein denaturation and the breakdown of protein compexes. He suggested that heat damage and cell death resulted directly from the latter two processes. He also noted a correlation between the heat resistance of proteins and their complexes, and the heat resistance of the cells from which they were obtained.

It is quite possible that when heat death occurs at high temperatures, protein coagulation is involved. However, heat coagulation is not usually thought of an occurring at lower temperatures, which also may be lethal to the intact organism. Indeed, Ushakov (1964) has pointed out that most protein preparations tested until that date, possessed a greater degree of thermostability than the cells or organisms from which they were isolated. Presumably, the denaturation process of these proteins can have no adaptive or compensatory significance. Ushakov (1964) suggests instead that the heat resistance of a cell is determined primarily by the thermostability of its least resistant protein, although what these are remains obscure.

Read (1967) has urged caution when relating heat resistance of organisms to the thermostability of their proteins, unless the temperatures at which protein activity is lost coincides closely with those at which metabolism fails. In only a few cases have these criteria been met. For example, myosin ATPase from the muscles of desert lizards (Licht, 1964), an amylase from a thermophilic bacterium (Manning and Campbell, 1961) and the

Mg^{2+}-dependent ATPase from the sarcolemma of freshwater crayfish (Bowler and Duncan, 1967; Bowler, Gladwell and Duncan, 1973; Gladwell, 1973).

Recently, Rosenburg, Kemeny, Switzer and Hamilton (1971) have provided thermodynamic evidence implicating the denaturation of proteins in thermal death of microorganisms. They found that the activation enthalpy (ΔH^*) and the activation entropy (ΔS^*) for protein denaturation were related by an empirical 'compensation law' of the form

$$\Delta S^* = a.\Delta H^* + b$$

The values for the constants a and b for a virus, yeasts and bacteria were closely similar to the values obtained for protein denaturation. It was suggested that this relationship gives good quantitative support for the general hypothesis that protein denaturation is the cause of heat death. However, Banks, Danjanovic and Vernon (1972) and Evans and Bowler (1973) have separately questioned the validity of this approach to multicellular organisms Evans and Bowler (1973) obtained somewhat different values for a and b for a variety of multicellular organisms and for the denaturation of proteins.

The most recent interpretation of the involvement of protein denaturation in resistance phenomena regard denaturation as any process by which one or more properties of the protein are reversibly lost (Sizer, 1943; Stearn, 1949; Johnson, Eyring and Polissar, 1954). This concept is supported by the recent suggestion of Hochachka and Somero (1973) that enzymes may become functionally inactive at temperatures well below their denaturation temperature, as a consequence of temperature-dependent changes in enzyme-substrate affinity. Heat death may, therefore, be caused by cessation of enzyme activity and all that this implies, rather than an irreversible change in the 3-dimensional structure of the protein molecule that is characteristic of coagulation.

In contrast to these ideas of a general breakdown in the metabolic processes of an organism during heat injury, are the conclusions of Bowler (1963b). He compared the oxygen uptake of isolated tissues from normal and heat-killed crayfish and found that tissues from the latter respire at 35°C at levels not markedly different from the control, except in the hepatopancreas and nervous tissue, where there was a statistically significant reduction of 32% and 27% respectively. These results indicate that in the freshwater crayfish at least, heat death cannot be ascribed to a general failure of metabolism (with the possible exception of the hepatopancreas and nervous tissue), since this important physiological process proceeds under conditions which would rapidly cause the death of the intact organism. Similar conclusions were reached by Grainger (1969), in the mollusc Arianta.

Heilbrunn (1924) first drew attention to the correlation of the thermal sensitivity of various organisms with the melting points of their constituent fats. Later Bělehrádek (1935, 1957) enlarged upon this and formulated the 'lipoid liberation theory', which postulated that cellular heat injury is caused by the 'melting' of the lipid constituents of cells or cell membranes. This theory links the heat resistance of an organism with the 'melting' of its fats which depend in large part upon the degree of saturation of their constituent fatty acids. Indeed, Bělehrádek (1963) later stated that "fats and phospholipids are the only protoplasmic constituents in which molecular structure and resulting physico-chemical behaviour depend upon the temperature of formation". The possibility of similar modifications to the isoenzyme complement of certain cells has recently been considered by Hochachka and Somero (1973), although Bělehrádek (1963) thought this possibility unlikely "in view of the template being genetically fixed".

Earlier hypotheses such as these were erected in ignorance of the functional properties and subcellular location of the phosphatides. Indeed, these workers often do not differentiate between the depot fats and the membrane lipids either in their experimentation or consideration. Current concepts of the physico-chemical characteristics of membrane lipids are comparatively well developed, and it is evident that most animal membranes are normally in a liquid-crystalline state. Thus raising the temperature will not cause a 'melt' per se, since they are already fluid. However, the increased molecular motion of membrane components caused by a rise in temperature will have important and perhaps deleterious consequences for their functional properties, such as membrane permeability (see Chapters 2 and 3) and their effect on the activity of membrane anzymes (see Chapter 7).

It has been known for some time that acclimation to low temperatures leads to the incorporation of increased proportions of unsaturated fatty acids in the phospholipids of cellular membranes, (Johnston and Roots, 1964; Roots, 1968; Kemp and Smith, 1970) although few studies have attempted to correlate this phenomenon with resistance adaptation. Frankel and Hopf (1940) reared two species of blowfly at low and high temperatures. The phosphatides extracts from both species reared at high temperature were more saturated than those extracted from flies acclimated to lower temperatures. This phenomenon was correlated with an increased resistance to lethal high temperatures in the warm acclimated animals. However, the fact that two closely allied species, bred at the same temperature and possessing an identical unsaturation of membrane lipids, have different resistances to high temperatures was considered by these authors to indicate that the physical breakdown of these substances cannot be the direct cause of heat injury. House, Riordan and Barlow (1958) have also demonstrated

a good correlation between the heat sensitivity of Pseudosarcophaga affinis larvae and the degree of saturation of the dietary lipids.

Experiments with goldfish have shown a similar correlation between the acclimation temperature of the animal and the degree of saturation of its brain lipids (Johnston and Roots, 1964). These workers concluded that acclimation involves the ability to control the degree of membrane unsaturation in order to maintain a specific degree of membrane 'fluidity'. Data such as these are often interpreted in terms of 'capacity adaptation' (Roots 1968 Kemp and Smith, 1970), and the contribution of such biochemical changes to resistance adaptation is not considered.

On the other hand, Ushakov (1964, 1966) quotes work in which the lipid composition of muscle from Rana temporaria and Calliphora erthrocephala was altered without affecting the thermostability of the muscle fibres. Furthermore, a decrease in the thermostability of frog muscle was not associated with a change in the nature of the lipid constituents. He concluded that the available evidence indicated no definite correlation between irreversible cell damage and the melting points of the lipid constituents.

A novel suggestion is that of Bowler, Duncan, Gladwell and Davison (1973) that cellular heat injury and heat death is a property of membranes, but is not necessarily related solely to the phospholipid or protein fraction, but rather to the stability of lipoprotein complexes or of enzymes whose activity is dependent upon the maintainance of membrane integrity.

Bowler (1963a) and Gladwell (1973) have demonstrated that Austropotomobius pallipes possesses a considerable degree of reasonable resistance adaptation. Heat death of the animal has been correlated with a rapid increase in haemolymph K^+ and with a simultaneous loss of Na^+. These workers suggested that this phenomena was caused by a dramatic breakdown in the passive permeability barriers to these ions, resulting in their exchange between the intra and extra-cellular compartments (Bowler and

Duncan 1967). It was later suggested (Bowler and Duncan 1967; Bowler, Duncan and Gladwell 1973; Gladwell 1973) that since these changes are large, a bulk tissue such as muscle was involved.

In a parallel series of experiments, the effects of exposure to lethal temperatures upon the resting potential of abdominal extensor muscle fibres was determined in 10°C and 25°C acclimated crayfish. In both cases, there was an initial small hyperpolarisation of the resting potential. However, whilst the resting potential of the 10°C acclimated crayfish declined steadily, the 25°C acclimated muscle preparation maintained the slight hyperpolarisation for over 20 minutes before steadily depolarising. Evidently, the membrane preparation from 25°C acclimated crayfish was more resistant to the deleterious effects of high temperature than the 10°C acclimated preparation. Death itself was due to the effects of high haemolymph K^+ concentrations upon the nervous system (Gladwell, unpublished observations). Indeed, these workers have found a correlation between the breakdown in passive permeability control of the muscle membrane and the temperature sensitivity of a membrane-bound Mg^{2+}-dependent ATPase isolated from the abdominal muscle of the crayfish. All these phenomena occurred over a limited temperature range (30-35°C) and were affected by acclimation in an adaptive manner.

Similar extensive lowering of haemolymph Na^+ has also been demonstrated in Arianta (Grainger, 1969), Centrioptera (Ahearn, 1970), Helix (Grainger, 1973a) and frog gastrocremius (Grainger 1973b). Grainger (1973b) has also noted a drop in the resting potential of frog muscle during exposure to temperatures that are lethal to the intact animal. It is remarkable that such dissimilar animals as those noted above should show similar reactions to lethal high temperature stress, and suggests that views expressed by Bowler and his colleagues may also apply to a wide variety of organisms.

Other workers have also indicated that heat injury may be associated with damage to cellular membranes. Davison and Bowler (1971) have shown that sarcosomes from the thorax of Calliphora erythrocephala have an equivalent thermal sensitivity to that of the whole animal. In addition, Davison (1971) has described ultrastructural changes in mitochondria from blowfly flight muscle following exposure to lethal temperatures. These workers concluded that the loss of mitchondrial function through disruption of membrane structure in this animal is an important factor in heat death.

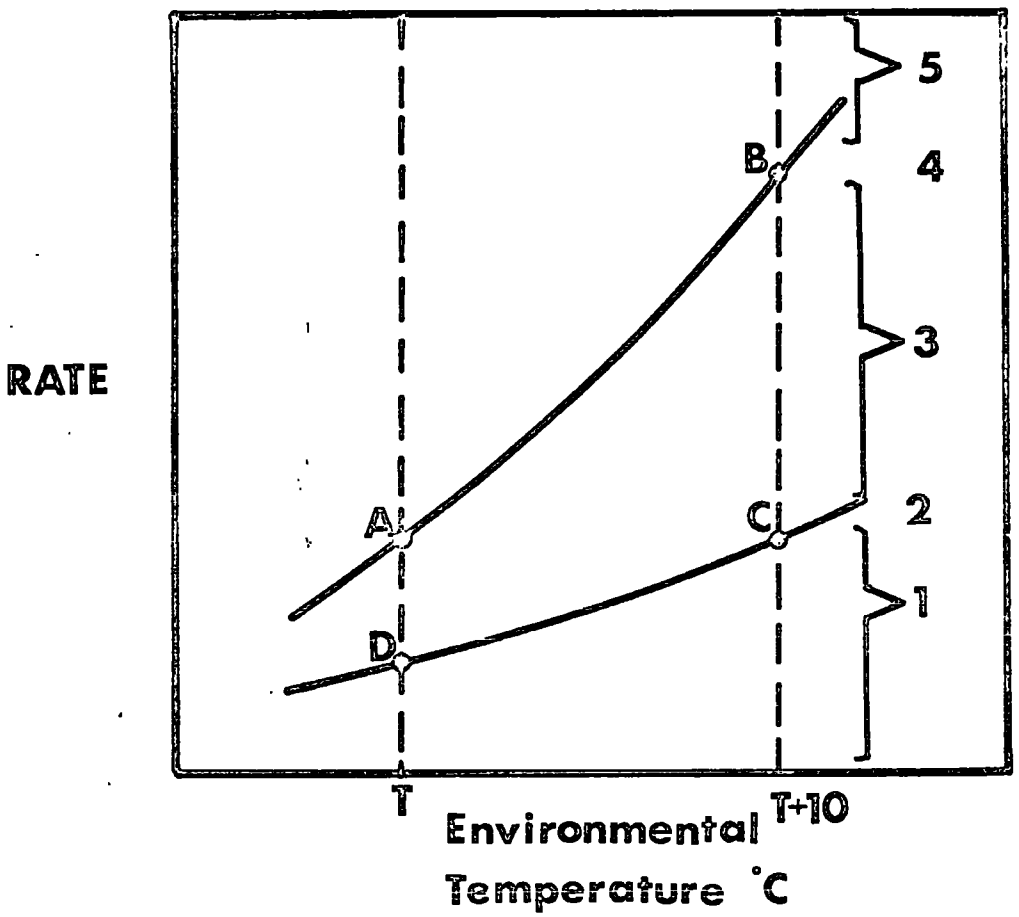
Iandola and Ordal (1966) have shown that heat damage to Staphylococcus aureus is caused by an increase in the permeability of the cytoplasmic membrane and a consequent leakage of solutes. A reduction in activity of respiratory enzymes after heat treatment of the whole animal was also observed by Bluhm and Ordal (1969). Levy, Gollon and Elliot (1969) have observed ultrastructural changes in organelles from Tetrahymena sp. following hyperthermia which, they suggest, reflects damage to membranes. Ling (1967) has reported a dramatic change in the permeability of frog striated muscle sarcolemma to sucrose, which occurs over a narrow temperature range of 35-40°C, which corresponded to lethal temperature.

The recent involvement of cellular membranes in both resistanc and capacity compensation indicates that these structures have a significance to these processes which matches their undoubted physiological importance. The experiments described in this thesis were designed to investigate the role of cellular membranes in thermal acclimation by comparing some physiological and biochemical characteristics of membranes isolated from animals acclimated to different temperatures. The conclusions of Bowler and his colleagues (Bowler 1963a; Bowler and Duncan, 1967; Bowler Duncan, Gladwell and Davison, 1973; Bowler, Duncan and Gladwell,

1973; Gladwell, 1973) that the primary lesion of heat death in the freshwater crayfish Austropotomobius pallipes occurs in the sarcolemma, together with the demonstrated influence of thermal acclimation upon the kinetics of heat death, the thermostability of certain neurophysiological characteristics of the sarcolemma, and the Mg^{2+}-ATPase, suggest that the muscle membrane is intimately involved in the process of resistance adaptation in this species. It is the purpose of this thesis to examine the muscle membranes in more detail to discover which component(s) is modified during acclimation and the effect of this modification to its functional characteristics.

In the first instance, the lipid biochemistry of muscle membranes was studied in some detail and the effect of thermal acclimation upon the membrane phospholipids assessed (Section I). Secondly, the Ca^{2+}-stimulated ATPase (E.C. 3.6.1.3), associated with the sarcoplasmic reticulum of crayfish muscle was used as a convenient 'model' membrane enzyme with which to study the effects of acclimation and an altered lipid environment upon the kinetic properties of an intrinsic membrane-bound enzyme (Section II). Thirdly, the thermal inactivation characteristics of this microsomal enzyme were studied in order to seek a correlation with the heat death kinetics of the intact animal, and to mimic the process of resistance compensation in vivo.

FIGURE 1-1



Precht's Classification for Capacity Compensation

(See text ; Precht, Christophersen, Hensel & Larcher 1973)

Section I

STUDIES ON THE BIOCHEMICAL COMPOSITION OF MUSCLE MEMBRANES

"Phosphatides are the centre, life, and chemical soul of all bioplasm whatsoever, that of plants as well as animals. Their chemical stability is greatly due to the fact that their fundamental radicle is a mineral acid of strong and manifold dynamicities. Their varied functions are the result of the collusion of radicles of strongly contrasting properties. Their physical properties are, viewed from a teleological point of standing, eminently adapted to their functions. Amongst these properties none is more deserving of further inquiry than those which may be described as their power of colloidation. Without this power no brain as an organ would be possible, as indeed the existence of all bioplasm is dependent on the colloid state."

J. L. W. Thudichum (1884).
The Chemical Constitution of the Brain
Bailliere, Tindall and Cox, London.

Chapter 2

THE EFFECT OF THERMAL ACCLIMATION UPON THE PHOSPHOLIPID AND CHOLESTEROL CONTENT, AND THE PHOSPHOLIPID COMPOSITION OF CRAYFISH MUSCLE MEMBRANES

INTRODUCTION

Studies on the gross chemical composition of cellular membranes have been handicapped by the inability to obtain pure membrane preparations. The problem of contamination of membrane preparations by membrane or non-membrane components is a recurring one. (For a critical evaluation of plasma membrane fractionation techniques, the reader is referred to Fleischer and Rouser, 1965, and Wallach and Lin, 1973.) Criteria for the purity of isolated membrane preparations are usually based upon the presence or absence of suitable morphological, chemical or enzymatic 'markers', which distinquish the membrane system from other contaminants.

Bearing these limitations in mind, most plasma membranes, from animal sources, appear to contain approximately 35-40% (by weight) lipid, 50% protein, 5% carbohydrates and 0.1% RNA (O'Brien 1967, Rothfield and Finkelstein 1968, Veerkamp 1972).

Usually the lipid component is composed of approximately 70% (by weight) polar lipids and 30% neutral lipids (Veerkamp, 1972). These are usually phospholipids and cholesterol, respectively. Almost all theories concerning the ultrastructure of cellular membranes rely heavily upon a major structural role for phospholipids. The notion originally proposed by Gortner and Grendel (1925) and refined by Davson and Danielli (1943), suggested that phospholipids provided a 2-dimensional, bimolecular leaflet, with proteins absorbed onto both surfaces. However, recent work has indicated that

although the bimolecular leaflet is probably an important structural element of membranes, it is interrupted to varying degrees by intrinsic membrane-bound protein molecules (Singer and Nicolson 1972; Vanderkooi, Senior, Capaldi and Hayashi 1972; Singer 1974).

An important argument in favour of a major structural and functional role for phospholipids in biological membranes is the behaviour of purified phospholipids in water or salt solutions. In the presence of excess aqueous phase, the phospholipids swell spontaneously to form a series of concentric, bimolecular membranes ('liposomes'), each separated by an aqueous compartment (Bangham 1968). The use of liposomes as a model system for permeability studies has been described by Hill and Cohen (1972).

It has recently been demonstrated that such protein-free membrane systems can successfully mimic certain functional properties of biological membranes (De Gier, Haest, Van der Neut-Kok, Mandersloot and Van Deenen 1972; Van Deenen 1969). For example, the graded permeability of a number of non-electrolytes across the membranes of both natural and model systems are very similar (Bangham, De Gier and Greville 1967; Klein, Moore and Smith 1971), and are in good agreement with the correlation between membrane permeability and the coefficient of distribution at an oil-water interface, originally established by Overton. In addition, liposomes exhibit a similar temperature dependence of non-electrolyte permeability as do the natural membranes from which they were derived (Van Deenen 1969, McElhaney, De Gier, Van der Neut-Kok 1973). However, this should not be construed as meaning that the permeability properties of biological membranes are exclusively determined by the lipid bilayer, since the permeability coefficient of

pure phospholipid membranes is 10^2-10^3 lower than in natural membranes. This difference may be considerably reduced by the incorporation of basic protein into the former system (Kimelberg and Papahadjopoulos, 1971a, b; Calissano and Bangham, 1971).

Van Deenen (1972) has recently emphasised that the permeability of biological membranes depends upon several factors, including the nature of the polar 'headgroup' of the amphipathic lipid component. This has been demonstrated most effectively in microbial and model membrane systems where the packing characteristics and net change of membrane phospholipids have implications for the ion selectivity and degree of permeability displayed by the membrane (Hepfer, Lehninger and Lennarz, 1970).

A second factor which is important for the stability and permeability properties of membranes is their sterol content. Cholesterol occurs in biological membranes in highly variable concentrations. In mitochondrial and nuclear membranes the concentration is low, but in the outer cell membranes the concentration is normally high, with molar phospholipid : cholesterol ratios close to 1 (Van Deenen, 1965).

Monolayer studies have shown that cholesterol, when present in molar equivalents, has a marked effect upon the expanded film obtained with unsaturated lecithins producing a more condensed film and showing a decrease in the molar area per lecithin molecule (Van Deenen, Houtsmuller, De Haas and Mulder, 1962; Demel, Van Deenen and Pethica, 1967). One might expect this increase in membrane packing to be of importance to the permeability barriers of lipid bilayers. Indeed, the <u>in vivo</u> incorporation of cholesterol does result in a definite decrease in glycerol and erythritol permeability of intact <u>Acholeplasma</u>

laidlawii cells and their derived liposomes (De Gier, Mandersloot and Van Deenen, 1968; De Kruyff, Demel and Van Deenen, 1972; Demel, Guerts Van Kessel and Van Deenen, 1972). In addition, Bruckdorfer, Demel, De Gier and Van Deenen (1969) have demonstrated that cholesterol-depleted erythrocytes become more permeable to glucose and less stable to osmotic stress. Papahadjopoulos and Watkins (1967) have reported a decreased chloride permeability, and Papahadjopoulos, Nir and Okhi (1972) a decreased cation permeability for liposomes prepared from a mixture of egg-yolk lecithin and cholesterol, compared with those preparations from pure lecithin.

Cholesterol also causes a decrease in hydrocarbon chain mobility of various artificial and natural membranes using spin-label techniques (Hubell and McConnell, 1971), nuclear magnetic resonance spectroscopy (Chapman and Penket, 1966) and X-ray diffraction techniques (Wilkins, Blaurock and Engelman, 1971). The incorporation of increasing amounts of cholesterol into phospholipid bilayers reduces and finally abolishes the thermotropic phase transition of the phospholipids, as observed by differential scanning calorimetry (Ladbrooke and Chapman, 1969; De Kruyff, Demel and Van Deenen, 1972). In addition, Szabo, Eisenman and Ciani (1969) have demonstrated that the presence of cholesterol in black lipid membranes increases their electrical resistance. Thus cholesterol appears to promote a tighter packing of the phospholipid molecules, and the increased stability and decreased permeability of membranes is due to greater hydrophobic interactions between adjacent hydrocarbon chains.

In view of the important roles that both phospholipids and cholesterol have in the structural and functional characteristics of biological membranes, it was of some importance to determine whether there were any changes in the amounts of

these membrane components during thermal acclimation. The experiments described in this chapter were designed to compare the total phospholipid and cholesterol content, together with the phospholipid class distribution of lipid extracts from the abdominal muscle of crayfish acclimated to 4°C and 25°C.

MATERIALS AND METHODS

A. MATERIALS

1. Chemicals. The following reagents were obtained from British Drug House Chemicals Ltd. (Poole, Dorset), and were 'AnalaR' grade: KH_2PO_4; $(NH_4)_6Mo_7O_{24}.4H_2O$; $FeCl_3.6H_2O$; $CuSO_4.5H_2O$; $K_2Cr_2O_7$; Hydrochloric Acid (35% w/v), Formic Acid (98% w/v), Sulphuric Acid (98% w/v), Nitric Acid (69-72% w/w), Perchloric Acid (71-73% w/v), Acetic Anhydride (97%), Glacial Acetic Acid (99.7% v/v), Chloroform, Methanol, Petroleum Ether (40-60°C b.p.), Acetone, Ninhydrin (indanetrione hydrate) and iodine (resublimed). p-xylene-2-sulphonic acid (2,5-dimethyl-benzene sulphonic acid) (laboratory grade), was also purchased from B.D.H. Chemicals Ltd. Resorcinol and Rhodamine 6G were supplied by Hopkin and Williams Ltd. (Romford, Essex). Fiske and Subbarow Reducer, Butylated Hydroxytolene (BHT, 2,6-ditert-butyl-p-cresol) and 2,4-dinitro-phenylhydrazene were purchased from the Sigma London Chemicals Co. Ltd. (Kingston upon Thames, Surrey). Bismuth III nitrate (basic G.R.) and Kieselgel H, Type 60 for Thin-Layer Chromatography were purchased from E. Merck (Darmstadt, West Germany).

2. Authentic Phospholipids. Sphingomyelin (ex-bovine brain, pure), phosphatidyl inositol (ex-yeast, pure) and cardiolipin (ex-ox heart) were purchased from Koch-Light Laboratories (Colnbrook, Buckinghamshire). Egg Lecithin (grade I), lysolecithin (grade I), phosphatidyl ethanolamine (egg, grade I) and phosphatidyl serine (bovine, acidic form, grade I) were obtained from Lipid Products (Nutfield Nurseries, South Nutfield, Nr. Redhill, Surrey). Cholesterol standard solution and lipid standards 178-1 and 178-3 were purchased from the Sigma London Chemical Co. Ltd. (Kingston upon Thames, Surrey).

3. Animals. Crayfish (Austropotambius pallipes, Lereboullet) were caught by hand in streams, or by trap ('Eclipse' Sparrow Trap, S. Young and Sons Ltd., Misterton, Somerset) in ponds of the Whittle Dene, Hallington and Matfen reservoir complexes near Corbridge, Northumberland. They were maintained until required in the laboratory at 15°C and 18 hour-light photoperiod, in stainless steel tanks (0.30 x 0.90 x 0.15m) filled with clean tapwater. Efficient aeration of the water was vital to keep the crayfish in good condition, particularly if the density of animals was high. It was not necessary to dechlorinate the tapwater before use.

The crayfish were fed at 3-4 day intervals with Trouw grade I pellets (Trouw Co. Ltd., Harston, Cambridge). Crayfish undergoing moult were placed in separate wire cages within

the tank to prevent cannibalism. Each tank was provided with broken flower pots and bricks for crayfish to hide beneath.

4. Glassware. Glassware was good quality borosilicate glass and obtained from J. A. Jobling and Co. Ltd. ('Pyrex' and 'E-Mil Gold Line' Brands) and A. Gallenkamp Ltd. (Technico Brand). Glassware for rotary evaporation was purchased from Buchi Ltd. Soda glass ampoules (2ml capacity) were purchased from Baird and Tatlock Ltd.

B. METHODS

1. Acclimation of Crayfish. Crayfish were kept at $4 \pm 0.5^{\circ}C$ or $25 \pm 0.1^{\circ}C$ in stainless steel tanks, filled with clean aerated tapwater for at least 21 days before sacrifice. (Bowler (1963a) has concluded that acclimation as measured by a change in thermal resistance is complete within 3 days at $25^{\circ}C$ and 8 days at $8^{\circ}C$.) The water in the $25^{\circ}C$ tank was circulated by a 'Circon' water pump (Baird and Tatlock Ltd.) and heated with a 500 watt immersion heater controlled by a 'Jumo' electrical contact thermometer (Gallenkamp Ltd.) and a Type F102-4 hot wire vacuum switch relay (Sunvic Controls Ltd.). An 18 hour-artificial light daylength was provided at both acclimation temperatures by fluorescent lights controlled by a time switch.

2. Treatment of Glassware. Glassware was soaked overnight in 50% aqueous HNO_3, then washed six times in tapwater followed by six times in distilled water. All items were oven dried, except chromatography tanks and plates which were left to drain at room temperature.

3. Extraction of the Total Lipid from Crayfish Muscle.

The procedure followed was essentially that of Folch Lees and Sloane-Stanley (1957) modified according to the suggestion of subsequent workers (Rouser, Kritchevsky and Yamamoto, 1967). The abdominal flexor and extensor muscles of crayfish were rapidly dissected out, blotted dry, weighed and placed in $20cm^3$ chloroform-methanol (2 : 1 v/v), containing 0.005% (w/v) butylated hydroxytoluene/^{gm}wet weight of muscle.

The muscle was thoroughly broken up for 10 minutes using a Vortex Waring Blender (M.S.E. Ltd.) fitted with a stainless steel blade rotating at approximately 3,000 r.p.m. The suspension was homogenised further using an all-glass hand homogeniser (Gallenkamp Ltd.), and then allowed to stand under an atmosphere of pure dry nitrogen for approximately 30 minutes. The homogenate was filtered by suction through a 'Scintaglass' filter (porosity 3, Gallenkamp Ltd.), mounted on a glass adapter, fitted with a side-arm connected to a water-pump (Figure 2-1). The red filtrate passed directly into a rotary evaporator flask. The whole apparatus was enclosed in an atmosphere of pure dry nitrogen.

The filtrate was re-extracted by resuspension in 10 cm^3/gm. wet weight of original muscle, of each of the following media for 15 minutes at room temperature followed by filtration as described above:

(1) Chloroform-Methanol (2 : 1, v/v)

(2) Chloroform-Methanol (1 : 1, v/v) with 1% concentrated HCl.

(3) Chloroform-Methanol (1 : 1, v/v) with 0.5% ammonia

The pooled filtrates were dried down to approximately 80mls in a Rotary Evaporator 'R' (Buchi Ltd.) at 25°C. The extracted lipids in organic solution were shaken thoroughly with 0.2 volumes (i.e., 16 mls) 0.79% KCl solution to extract non-lipid contaminants (Folch, Lees and Sloane-Stanley, 1957). The emulsion was allowed to settle and complete separation of the organic and aqueous phases was ensured by centrifugation in a 'Mistral 2L' centrifuge (M.S.E. Ltd.) at 1000g using a swing-out head (Rotor No. 69166) fitted with 100ml glass centrifuge tubes. The organic phase was removed with a Pasteur pipette and dried down to approximately 2ml in the rotary evaporator. Residual water was removed using the solvent replacement technique of Rouser, Kritchevsky and Yamamoto (1967). Finally, the concentrated lipid extract was placed in a glass vial. Lipids were stored under an atmosphere of pure nitrogen in a sealed glass vial and placed in the dark at -20°C until required.

4. Prevention of Autoxidation. The lipid extracts were protected against autoxidation during extraction and storage by rigorously adhering to the following procedures (Holman 1966):

   a. The use of 0.005% 4-methyl-2,6-di-tert-butylphenol (butylated hydroxytoluene) as antioxidant in the initial extraction medium, in chromatographic solvents and in the initial eluting solvents, as suggested by Wren and Szczepanowka (1964).

   b. Lipid extracts were maintained under an atmosphere of pure dry nitrogen gas wherever possible. Oxygen was excluded from the extraction and storage media by bubbling with nitrogen gas after use. After rotary evaporation the vacuum was discharged with nitrogen gas.

   c. Lipids were stored at -20°C in the dark under pure nitrogen gas in sealed glass ampoules.

5. Thin layer Chromatography

   a. Preparation and Washing of Silica Gel (Parker and Peterson, 1965) 250g Silica Gel was washed in 500mls chloroform/methanol/formic acid (1 : 2 : 1) by stirring vigorously with a glass rod, in order to remove inorganic contaminants. After 10 minutes the "fines" were decanted together with excess solvent. The formic acid solvent was removed by resuspending the residue in 500mls chloroform/methanol (1 : 1), allowing the silica gel to settle, and decanting the excess solvent. This procedure was repeated once

with 500mls chloroform and twice with distilled water. Remaining silica gel was dried thoroughly in an oven at 105°C for several hours. The lumps were carefully broken up using a ceramic mortar and pestle, followed by vigorous stirring using a Vortex Waring Blender.

b. Preparation of Thin Layer Plates. Glass Plates (200mm x 200mm x 3mm) were placed in a 'Unoplan' pneumatic holder (Shandon Scientific Ltd.) using plastic gloves, and thoroughly washed with chloroform. 30 grams of washed silica gel were suspended in 60mls of 0.01M potassium hydroxide by vigorously stirring for 5 minutes. The slurry was poured into a 'Unoplan' adjustable spreader with the aperture set at 250um using a feeler gauge. The spreader was moved smoothly and without interruption across the surface of the glass plates. The plates were briefly vibrated against a 'Whirlymix' Vortex Mixer (Fison's Laboratory Apparatus, Loughborough) to ensure that an absolutely smooth surface was obtained. Finally the plates were dried overnight in a horizontal position. The slurry was sufficient to coat five plates with a 250um layer. For the preparation of 500um thick chromatoplates for preparative TLC, the slurry was sufficient to cover 2 plates.

c. Application of Sample. Plates were activated at 105°C for 1 hour before use. All subsequent operations were performed under dry nitrogen gas. After the plate was cooled, lipids were applied as a chloroform/methanol (2 : 1) solution using

a 25ul 'Terumo' microsyringe (Shandon Southern Scientific Co. Ltd.) with a squared needle-tip. For 2-dimensional chromatography, phospholipids were applied as a series of spots (10 x 3mm) about 15 - 25mm from the corner of the plate, taking care not to disturb the layer of silica gel. Loads of 300-500ug phospholipid (600 - 1000ug total lipid) were routinely applied for phosphate analysis. For the application of accurate aliquots of lipid solutions an 'Agla' Micrometer all-glass syringe (Burroughs-Welcome and Co. Ltd.) was used. The syringe was mounted on a 'Prior' manipulator and fitted with a 15mm stainless steel hypodermic needle (25 gauge, Cooper Needle Works, Perry Bar, Birmingham) with a squared tip.

d. Solvents and Development of Chromatoplates.

Plates were developed by ascending chromatography in large, moulded-glass tanks (Shandon Southern Scientific Co. Ltd.) lined with Whatman 1MM filter paper. For analysis of phosphate or fatty acids the tanks were flushed with nitrogen prior to introduction of the plate. The tanks were fully saturated with solvent vapour prior to development by vigorously shaking the tank and solvent, and allowing it to stand for 1 hour at room temperature The developing solvents were present as a shallow layer (approximately 10mm) at the bottom of the tank and were allowed to rise up the chromatoplate until the solvent front was approximately 20mm from the top of a plate.

Phospholipids were separated using a 2-dimensional system modified after Rouser, Simon

and Kritchevsky (1969). The plates were initially developed in chloroform/methanol/7N aqueous ammonia (230 : 90 : 15, v/v/v). The plates were then dried thoroughly for at least 15-20 minutes under flowing pure dry nitrogen, and developed at 90° to the initial axis of development in chloroform/methanol/acetone/ glacial acetic acid/distilled water (9 : 3 : 12 : 3 : 1.5). The plates were then removed from the tank and dried in a stream of pure dry nitrogen (Figure 2-2). Neutral lipids were separated by development in petroleum ether (40-60°C, b.p.) : diethyl ether : glacial acetic acid, (70 : 30 : 2) (Figure 2-3).

The retention values (relative to the solvent front) of authentic lipid standards in the three solvent systems are given in Table 2-1.

e. Detection of Lipids on Thin-Layer Chromatogram.

The following spray reagents were applied to the dried TLC plates after separation using a glass spray (T. W. Wingent and Co., Ltd., Milton, Cambridge) powered by compressed air. The reactions of authentic lipid standards obtained commercially to the various spray reagents are summarised in Table 2-1.

(i) General Non-destructive tests

Rhodamine. Stock solution : 0.5% (w/v) aqueous Rhodamine 6G was washed twice with an equal volume of hexane to remove organic impurities (Parker and Peterson, 1965). This solution was stable indefinitely at room temperature.

Spray Reagent: 0.005% (w/v) aqueous Rhodamine 6G. Procedure: Chromatoplates were sprayed until distinctly pink. Lipids appeared as yellow spots on a bright green background when viewed under short wavelength UV light (254nm or 350nm, Camag Universal UV Lamp). This technique was used mainly for preparative work.

Iodine Vapour. (Mangold and Malins, 1960; Sims and Larose, 1962). Dry chromatoplates were placed in an atmosphere of iodine for 10 minutes. All lipids took up iodine and became brown spots on a pale brown background. Iodine could be sublimed off by warming at 105°C.

(ii) General Destructive Tests

Sulphuric Acid - Dichromate Reagent (Privett and Blank, 1962).

Spray Reagent: Saturated solution of $K_2Cr_2O_7$ in 70% (v/v) sulphuric acid. This solution was stable indefinitely at room temperature. Procedure: Chromatoplates were sprayed lightly with the spray reagent and heated at 180°C for 1 hour. All organic components yielded a grey-black spot.

(iii) Specific Destructive Tests

Free Amino Groups

Spray Reagent: 0.25% (w/v) Ninhydrin and 1% glacial acetic acid in acetone, prepared freshly.

Procedure: The chromatoplate was sprayed thoroughly and heated at 105°C for 5-10 minutes. Lipids containing free amino-groups showed up as distinct red-violet spots on a pink background.

Plasmalogens (Rietsma, 1954)

Spray Reagent: 0.4% (w/v) dinitrophenylhydrazine in aqueous 2N HCl.

Procedure: Chromatoplates were sprayed lightly. Plasmalogens appeared as yellow or orange spots after slightly warming at 105°C.

Phosphate-containing Lipids (Vaskovsky and Kostetsky, 1968)

Solution I: 16gm $(NH_4)_6 Mo_7 O_{24}. 4H_2O$ was dissolved in 120ml distilled water.

Solution II: 40mls concentrated HCl and 10mls of mercury were shaken with 80mls Solution I for 30 minutes to give a dark red-brown solution. The granular mercury was filtered off.

Solution III: 200mls concentrated H_2SO_4 followed by Solution II was carefully added to the remainder of Solution I. The cooled mixture was diluted to 1l with distilled water. Stable for u to 6 months.

Procedure: Phosphate-containing lipids react immediately upon spraying to give blue spots. If necessary the blue colour was intensified by heating at 105°C for 10-15 minutes. Cholesterol and cholesterol esters also react to yield intense red-violet spots. The light

blue background was reduced with a light water spray.

Gangliosides

Spray Reagent: 40mls concentrated HCl,
5ml 2% (w/v) Resorcinol,
0.25ml 0.1M cupric sulphate,
4.75ml distilled water.

Prepared at least 4 hours before use. Stable for one week.

Procedure: The chromatoplate was sprayed liberally with the reagent and a clean glass plate was placed over the thin-layer to prevent evaporation of the spray reagent. Both plates were heated at 120°C for 20 minutes when all sugar-containing spots became a blue/black colour.

Choline-containing Lipids (Wagner, Hörhammer and Wolff, 1961)

Solution I: 1.7g bismuth subnitrate (III) in 100ml of 20% glacial acetic acid.

Solution II: 40g of KI in 100ml water

Spray Reagent: ("Dragendorff Reagent")
20ml Solution I and 5ml of Solution II were mixed with 70ml distilled water.

Procedure: The chromatoplates were sprayed with the spray reagent. Choline-containing lipids appear as orange or orange-red spots immediately or after gentle warming.

Cholesterol and Cholesterol Esters (Lowry, 1968)

Spray Reagent: 50mg $FeCl_3.6H_2O$ in 90ml H_2O
5ml glacial acetic acid
5ml concentrated H_2SO_4

Stable for 3 months at room temperature.

Procedure: Chromatoplates were sprayed with the spray reagent and heated at 105°C for 2-3 minutes. Cholesterol and cholesterol esters develop a red-violet colour.

6. Determination of Phosphate

Considerable difficulty was encountered in finding an adequate method for estimating inorganic phosphate in lipid samples. The methods of McClare (1971) and Rouser, Siakotos and Fleischer (1966) gave inconsistent results because loss of perchloric acid at high digestion temperatures (up to 50%) led to spontaneous colour formation (see Rhee and Dugan, 1967). Also the use of ascorbate as reducing reagent (McClare, 1971; Rouser, Siakotos and Fleischer, 1966) led to high blank values.

By contrast, concentrated sulphuric acid (Barlett, 1959) did not fume off significantly during digestion at 200°C. A few drops of concentrated perchloric acid were added prior to digestion to accelerate the digestion process. The use of Fiske and Subbarow Reducer led to negligible blank values (Barlett,1959; Parker and Peterson, 1965).

Standard phosphate solutions were diluted from a stock solution of 100ug phosphorus (as KH_2PO_4)/ml. Unknown samples as chloroform/methanol (2 : 1) solutions or silica gel TLC spots were arranged to contain less than 100ug phosphorus. The standards, blanks and unknown samples were placed into 150 x 25mm 'Pyrex' boiling tubes with 0.5ml concentrated sulphuric acid and 3 drops of 72% (w/v) perchloric acid from a Pasteur pipette. Addition of 3-4 acid-washed glass beads

(approximate diameter 3-5mm) reduced loss of material through spitting and bumping. They were refluxed at 180-200°C on an electric Microkeldahl digestion rack (A. Gallenkamp Ltd.) until after approximately 30 minute the solution became colourless. This latter operation was performed in a fume cupboard behind reinforced glass as a precaution against explosions caused by perchloric acid (McClare, 1972).

The tubes were cooled and 9.5ml of a solution containing 91 volumes of 0.26% (w/v) $(NH_4)_6\ Mo_7O_{24}.4H_2O$ and 4 volumes of Fiske and Subbarow reagent, was added. The mixture was agitated thoroughly and placed in a boiling water bath for exactly 10 minutes to accelerate development of the heteropoly blue colour (Bartlett, 1959).

After a subsequent 20 minutes at room temperature, during which silica gel was centrifuged at 1000g for 10 minutes in a 'Mistral 2L' centrifuge (M.S.E. Ltd.), the % transmission of the solution was read at 820nm on a Spectronic 20 Spectrophotometer (Bausch and Lomb Ltd.) equipped with an infra-red phototube and filter. The phosphate content of unknown samples was determined by reference to a calibration line (Figure 2-4) constructed by treating standard phosphate solutions as above. The presence of 0.1g silica gel H during digestion did not affect the response characteristics (see Figure 2-4). Recovery of authentic phosphatidyl ethanolamine from the chromatoplate after chromatography was between 96-101% of an identical sample applied directly to the boiling tube. Silica gel blanks yielded negligible colour.

7. Determination of Cholesterol

The amount of cholesterol present in total lipid extracts was determined by a technique based upon the 'Liebermann-Burchard' reaction, but modified for the direct estimation of cholesterol in blood plasma (Pearson, Stern and McGavack, 1953; Watson, 1960).

Reagents: A. 0.25M 2,5-dimethylbenzene-sulphonic acid in glacial acetic acid

B. 3 volumes of Acetic Anhydride
1 volume of Solution A
This reagent was stored in a darkened bottle at $4^{o}C$ for up to 12 months.

Procedure: In order to facilitate mixing, analyses were carried out in 150 x 25mm 'Pyrex' boiling tubes. All glassware was completely dry since water interferes with the reaction.

Lipid samples containing cholesterol were placed in boiling tubes, and the organic solvents were evaporated with nitrogen. 0.1ml glacial acetic acid was added to dissolve the cholesterol. Reagent blanks included 0.1ml glacial acetic acid only, whilst a standard cholesterol solution (2mg/ml glacial acetic acid) was diluted with glacial acetic acid to give 100, 200, 300 and 400ug Cholesterol/0.2ml

To each blank, standard and unknown, 2.5ml of Solution B was added and the mixture was shaken thoroughly and left at room temperature. After 10 minutes 0.3ml concentrated sulphuric acid was added. After a further 20 minutes the optical density of the samples were measured in glass cuvettes (10mm light path) against a distilled water blank at 600nm in a Hilger and Watts Ltd. Spectrophotometer. The cholesterol content of

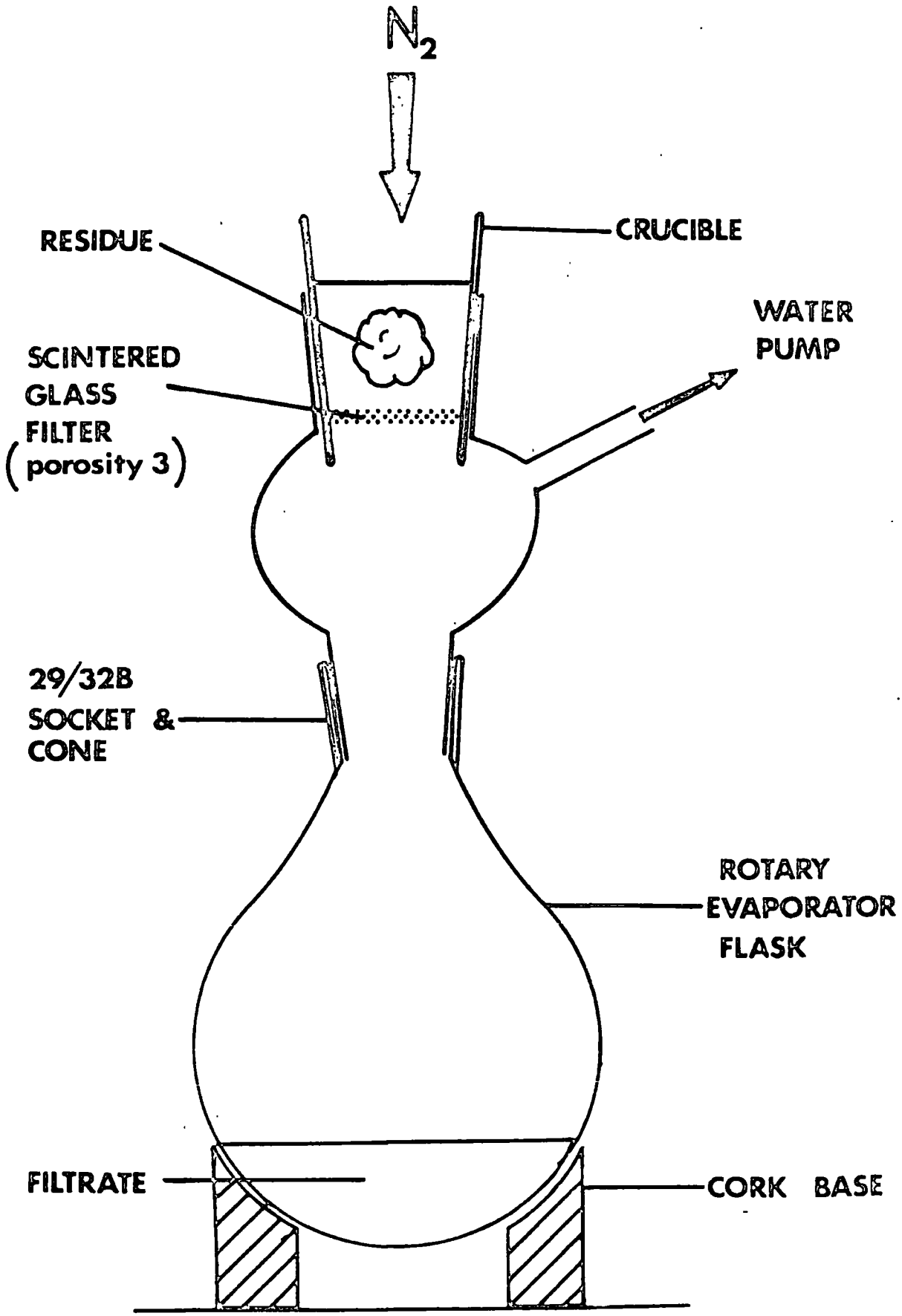


Figure 2-1: Diagram showing the Apparatus used for the filtration of tissue homogenates
The whole is enclosed under a plastic hood in an atmosphere of nitrogen gas

Figure 2-2: Photograph of a two-dimensional thin-layer chromatogram of phospholipids of a total lipid extract from crayfish abdominal muscle

Chromatogram: 0.25mm Silica gel H in 0.01M KOH

Solvent Mixtures: A Chloroform/Methanol/7M Ammonia (230 : 90 : 5, v/v/v)

B Chloroform/Acetone/Methanol/Glacial acetic acid/Water (90 : 120 : 30 : 30 : 15, by volume)

Visualisation: Sulphuric acid-dichromate spray reagent. See 'Materials and Methods'.

Identification of Components: The relevant data together with the tentative identities are presented in Table 2-2

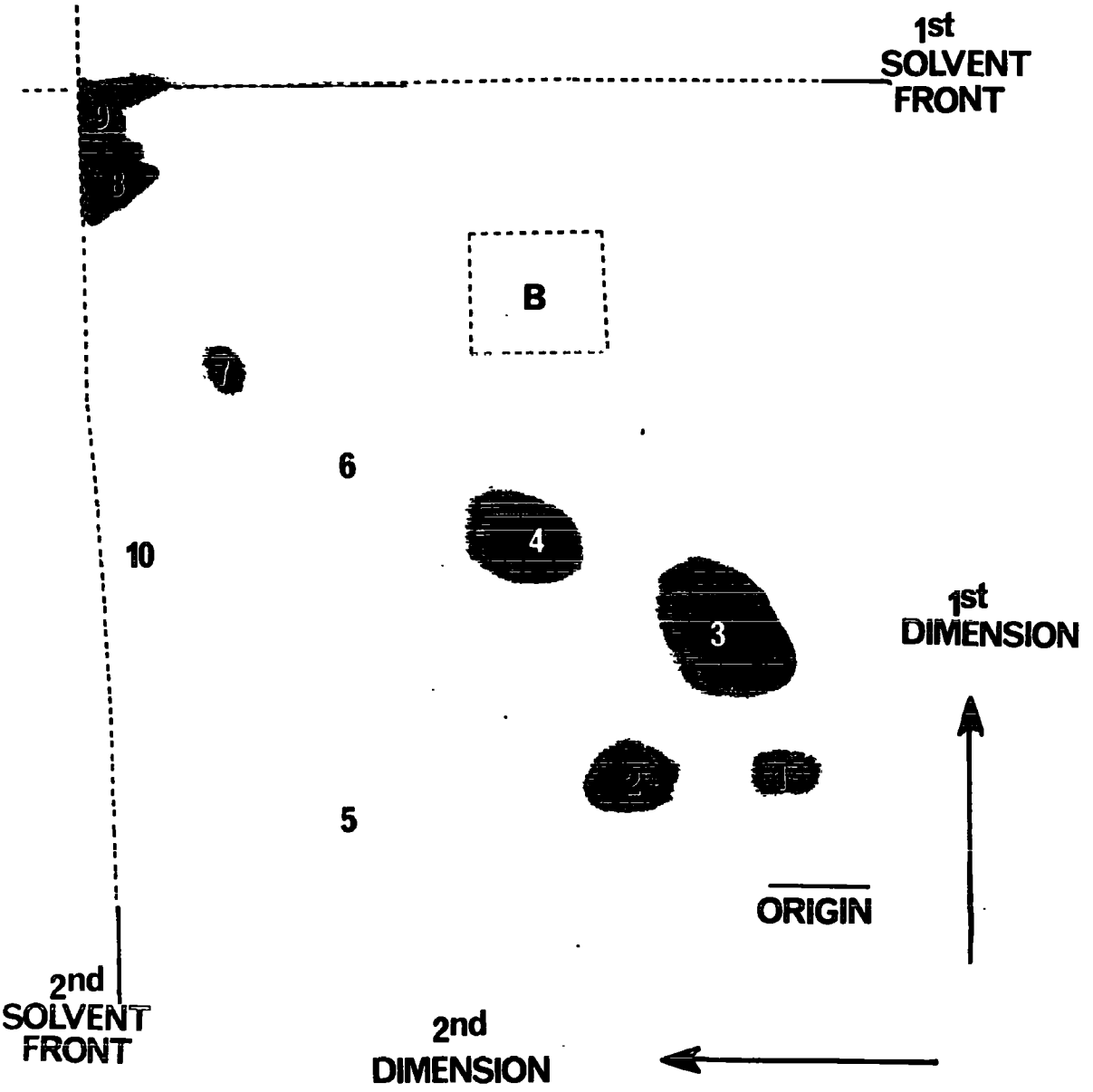

1st
SOLVENT
FRONT
9
8
B
7
6
10
4
3
1st
DIMENSION
2
1
5
ORIGIN
2nd
SOLVENT
FRONT
2nd
DIMENSION

Figure 2-3: Photograph of a one-dimensional thin-layer chromatogram of neutral lipids of a total lipid extract from crayfish abdominal muscle

Chromatogram: 0.25mm Silica gel H.

Solvent Mixture: Petroleum Ether (40-60°C b.p.)/ diethyl ether/glacial acetic acid (70 : 30 : 2, by volume)

Visualisation: Charring in an oven at 180°C for 1 hour, after spraying with sulphuric acid-dichromate reagent.

Identification of components: This was achieved by comparison with authentic lipids and reaction to specific spray reagents.

Legend: Mixture A - contains authentic lipids (Mixture 178-1, see 'Materials')

Mixture B - Crayfish muscle extract

Mixture C - authentic lipids (Mixture 178-3, see 'Materials')

1 - phospholipids

2 - monoglycerides

3 - cholesterol (and diglycerides?)

4 - fatty acids

5 - unknown

6 - fatty acid methyl esters

7 - triglycerides

8 - BHT

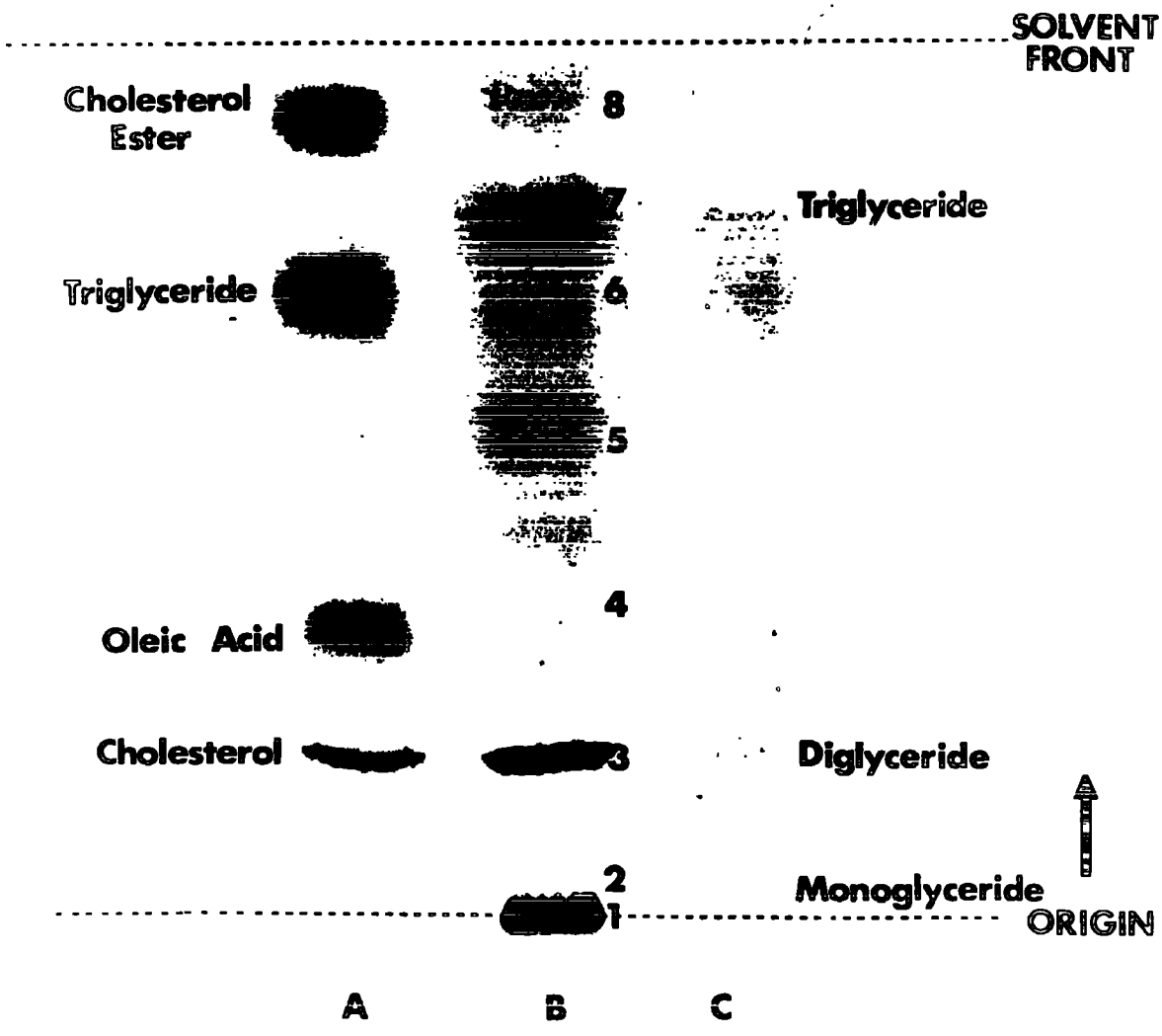

SOLVENT FRONT
Cholesterol Ester
Triglyceride
Oleic Acid
Cholesterol
8
7
6
5
4
3
2
1
Triglyceride
Diglyceride
Monoglyceride
ORIGIN
A
B
C

Figure 2-4: A typical calibration line for the determination of inorganic phosphate and the effect of silica gel H, on the development of colour

Methods: Phosphorus was analysed using a method modified after Bartlett (1959). Standard phosphorus solutions were diluted from a stock solution of 100ug Phosphorus (as KH_2PO_4)/ml.

The effect of silica gel on the reaction was studied by inclusion of 0.1gm silica gel H in each reaction tube. This was removed prior to reading by centrifugation.

Legend: Ordinate: % transmission at 820 nm

Abscissa: Amount of phosphorus (as KH_2PO_4) (ug)

● Average of duplicate analyses in the absence of silica gel H.

O Average of duplicate analyses in the presence of 0.1g silica gel H.

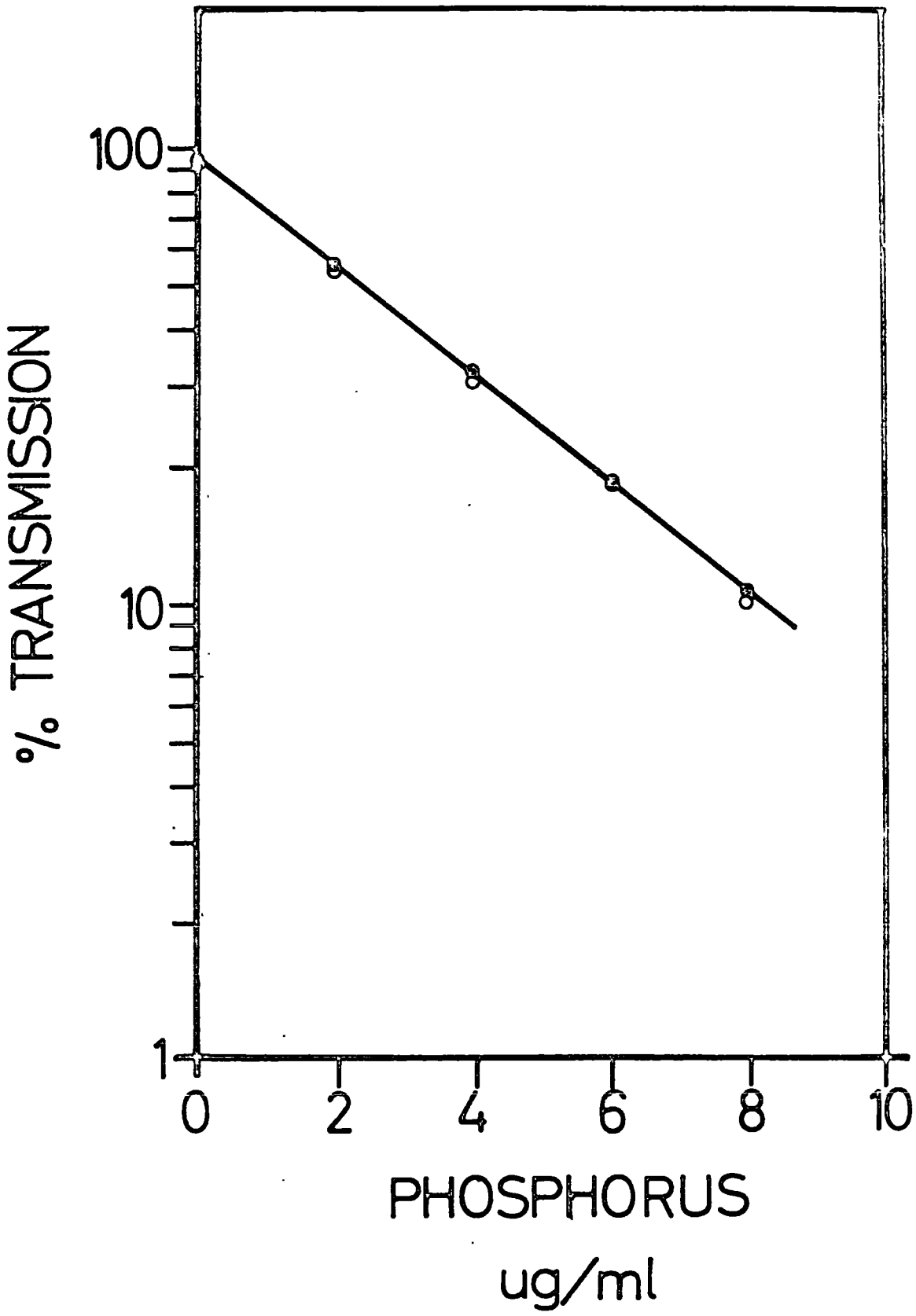

100
10
1
% TRANSMISSION
0
2
4
6
8
10
PHOSPHORUS
ug/ml

Figure 2-5a: A typical calibration line for the determination of Cholesterol

Method: Cholesterol content was determined using the method of Watson (1960). Standard cholesterol solutions were prepared by dilution from a stock solution containing 2mg cholesterol/ml glacial acetic acid. Values represent the average of duplicate analyses for each concentration.

Legend: Ordinate - Weight of standard cholesterol (ug)

Abscissa - Absorbance at 600 nm

Figure 2-5b: The effect of authentic phosphatidyl ethanolamine upon the spectrum of the cholesterol reaction

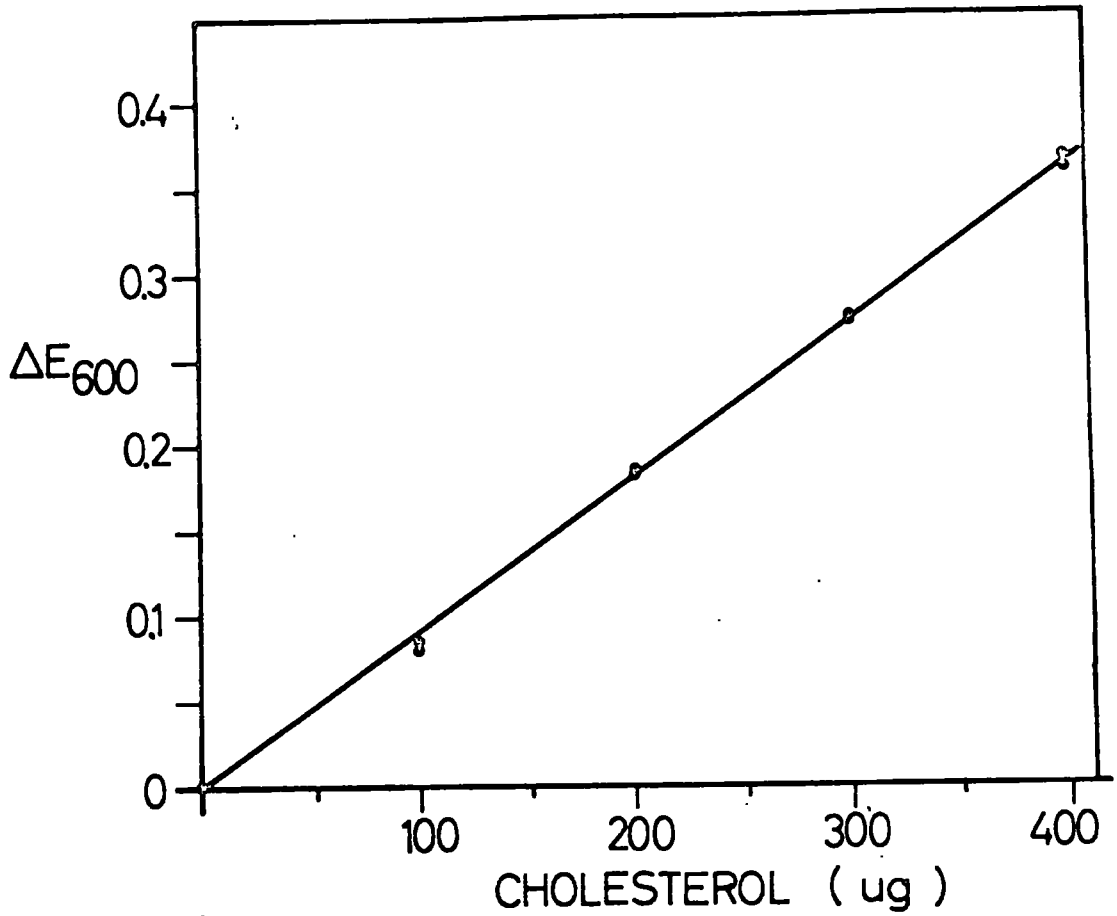
Method: The reagents and procedures are described in 'Materials and Methods'. Approximately 2.5mg of authentic phosphatidyl ethanolamine was included in one reaction tube with 250ug cholesterol.

Legend: Ordinate - Wavelength of incident light (nm)

Abscissa - Absorbance

● - Without phospholipid

○ - With phospholipid


0.4
0.3
ΔE_{600}
0.2
0.1
0
100
200
300
400
CHOLESTEROL (ug)

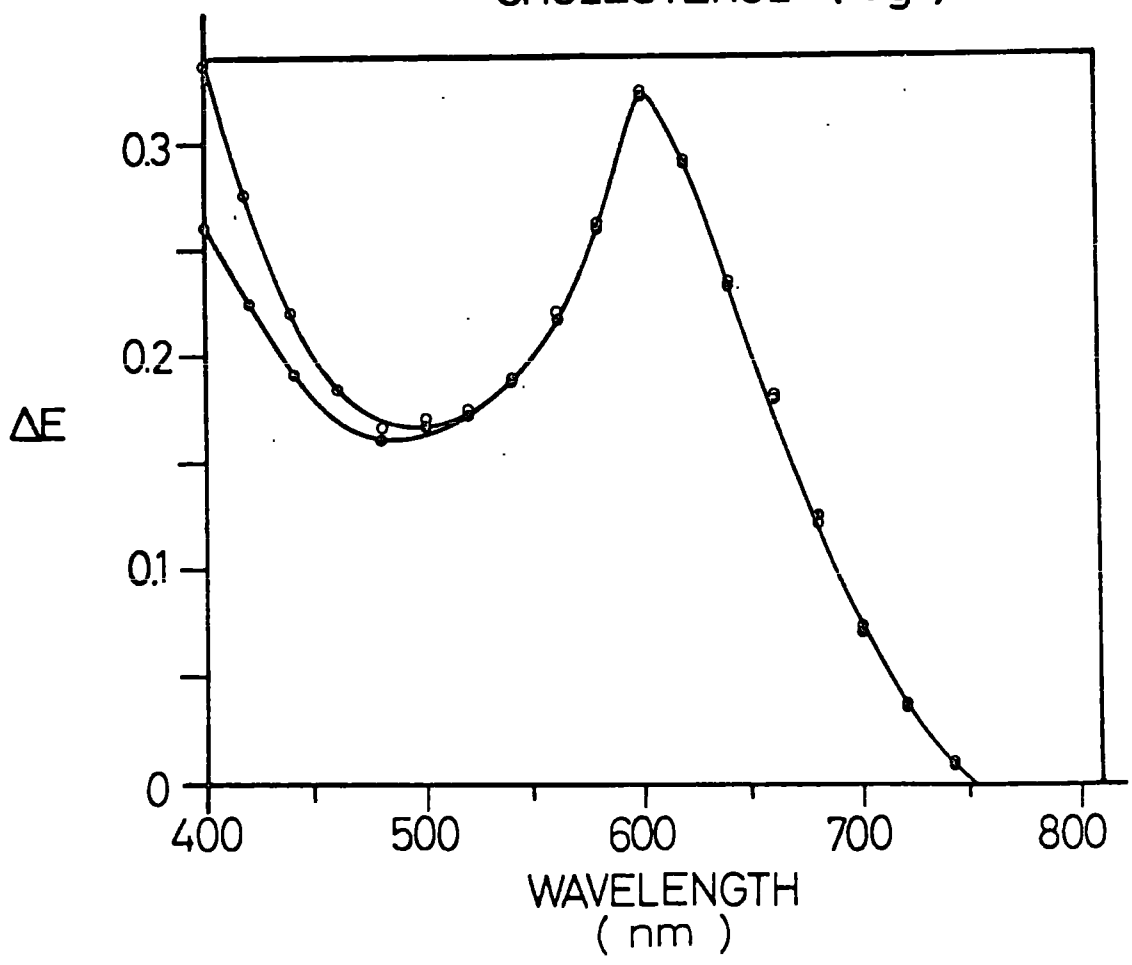

0.3
0.2
ΔE
0.1
0
400
500
600
700
800
WAVELENGTH
(nm)

Table 2-1: Summary of the chromatographic characteristics and reaction to various spray reagents of the authentic neutral and phospholipid standards

For details of spray reagents, and chromatographic techniques, see 'Materials and Methods'.

Legend:

Solvent System A - Chloroform : Methanol : 7N ammonia (230 : 90 : 15)

Solvent System B - Chloroform : Acetone : Methanol : Glacial Acetic Acid : Water (9 : 12 : 3 : 3: 1)

Solvent System C - Petroleum Ether (40-60°C, b.p.); Diethyl ether : glacial acetic acid (70 : 30 : 2)

* Rhodamine 6G

** Retention Distance (relative to the solvent front)

\+ - positively detected

\- - negative reaction

0 - not tested

Table 2-1

		GENERAL			SPECIFIC				RRf** Sol. A.	RRf** Sol. B	RRf** Sol. C
		Iodine	R6G *	Dichromate/ H_2SO_4 Spray	Phosphate	Resorcinol	Ninhydrin	Dragendorff Reagent			
Neutral Lipids	Triglycerides	+	+	+	-	-	-	-	0.96-1.00	0.98-1.00	0.79
	Diglycerides	+	+	+	-	-	-	-	0.96-1.00	0.98-1.00	0.16
	Monoglycerides	+	+	+	-	-	-	-	0.98-1.00	0.98-1.00	0.04
	Cholesterol	+	+	+	+	-	-	-	0.92-0.96	0.98-1.00	0.18
	Cholesterol Ester	+	+	+	+	-	-	-	0	0	0.89
	Fatty Acid (oleate)	+	+	+	-	-	-	-	0.96-1.00	0.98-1.00	0.31
	Oleate Methyl Ester	+	+	+	-	-	-	-	0.96-1.00	0.98-1.00	0.68
Polar Lipids	Sphingomyelin	+	+	+	+	-	-	+	0.14	0.08	0.00
	Phosphatidylcholine	+	+	+	+	-	-	+	0.31	0.13	0.00
	Lysophosphatidylcholine	+	+	+	+	-	-	+	0.07	0.03	0.00
	Phosphatidylserine	+	+	+	+	-	+	-	0.15	0.29	0.00
	Phosphatidylinositol	+	+	+	+	-	-	-	0.15	0.29	0.00
	Phosphatidylethanolamine	+	+	+	+	-	+	-	0.44	0.43	0.00
	Phosphatidic Acid	+	+	+	+	-	-	-	0.09	0.73	0.00
	Cardiolipin	+	+	+	+	-	-	-	0.52	0.75	0.00
	Gangliosides	0	0	+	0	+	0	0	0	0	0.00
	Background	Light Brown	Bright Green	White/ Grey	Blue/ White	White	Pink	White/ Yellow	-	-	-

unknown samples was determined by reference to a calibration line prepared from the standard solutions (Figure 2-5a).

Figure 2-5b shows the spectrum of the resultant blue solution with a peak at 600nm. The presence of large amounts (2.5mg) of phosphatidyl ethanolamine did not interfere with the peak between 520-740nm although it did cause an enhanced optical density at shorter wavelengths.

RESULTS

Identification of Lipids

Phospholipids were fractionated on KOH-impregnated chromatoplates using a 2-dimensional system as described in the methods. A typical separation is illustrated in Figure 2-3.

The identity of the separated components was determined tentatively using specific spray reagents and by co-chromatography and chromatographic comparison with authentic lipids obtained commercially. The results of this analysis are summarised in Table 2-2 together with the abbreviations for each phospholipid class used henceforth.

Each chromatographic spot consisted of only one phospholipid class except for spot number 2 which chromatographed with both serine and inositol phosphoglycerides. In spite of claims to the contrary (Rouser, Simon and Kritchevsky, 1969; Erdahl, Stolyhwo and Privett, 1973) it did not prove possible to separate these phospholipids with the solvent systems employed. Gas-liquid chromatography of the trimethylchlorosilane derivatives of the acid hydrolysis products of spot number 2 did not yield a peak that corresponded with either the TMS derivatives of authentic meso-inositol (B.D.H. Chemicals Ltd.) or the acid hydrolysis products of authentic phosphatidyl inositol (D. Winterbourne,

personal communication). Thus, whilst the positive reaction with ninhydrin confirms the presence of PS, there is no direct evidence that PI is also present.

Spot number 7 (see Table 2-2 and Figure 2-2), although positively identified as a phospholipid, did not co-chromatograph with any of the available authentic phospholipids. It was tentatively identified as a cerebroside by reference to the chromatographic data presented by Rouser, Simon and Kritchevsky (1969).

Spot number 10 (see Table 2-2 and Figure 2-2) was phosphate negative and was thought to consist of free fatty acids, perhaps originating from PE by cleavage at the beginning of development with the second acetic acid-containing solvent system.

It is important to remember that although each spot contains one phospholipid class (except perhaps PS/PI), the positive plasmalogen reaction observed with PC, PE and PS/PI (Table 2-2) confirms that they are probably a complex mixture of phosphoglycerolipid analogues containing acyl, alkyl and alk-1-enyl linkages between the glycerol and the hydrocarbon chain. Since chromatographic techniques for the separation of these mixtures are not easily available (Snyder, 1973) the phospholipid classes were not fractionated further.

Neutral lipids were separated and identified using a one-dimensional system as described by Skipski and Barclay (1969). A typical separation is illustrated in Figure 2-3. Separated components were tentatively identified by specific spray reagents (see Table 2-2) and by comparison with authentic lipids obtained commercially. Phospholipids remained at the origin but were not completely separated from the monoglyceride fraction.

The sterol content of crayfish muscle is exclusively cholesterol since cholesterol esters were not detected in the

muscle extract. Cholesterol and diglycerides were not separated using this solvent system. Astaxanthin, a red pigment derived from the denaturation of a xanthoprotein present in muscle, moved with the solvent front.

The Effect of Thermal Acclimation on Phospholipid Composition

Phospholipids were separated using a 2-dimensional TLC system as described previously (see 'Methods'). The separated components were visualised with iodine vapour and scraped into 150 x 25mm 'Pyrex' boiling tubes. The lipids were digested with a concentrated sulphuric acid-perchloric acid mixture and the amount of inorganic phosphate liberated from each component was determined as described in the 'Methods'. The amount of phosphate in each separated component was expressed as a percentage of the total phosphate recovered from the chromatoplate This calculation effectively gives a mole % composition, since each phospholipid identified (except cardiolipin, a very minor component) contains only one phosphate moeity per lipid molecule.

The phospholipid composition of the lipid extracted from the abdominal muscle of each animal was analysed separately several times and the mean phospholipid (mole %) composition together with the range of values encountered, is presented in Table 2-3 for total muscle extracts from 4°C-acclimated crayfish and Table 2-4 for total muscle extracts from 25°C-acclimated crayfish.

There was some small variation in the results of replicate analyses of each muscle lipid extract, but this is to be expected since an error in the phosphate determination of one component automatically affects the final percentage results of all other components. In addition, some variation was observed between the mean phospholipid composition of different crayfish with the same acclimation history. This variation may be due to a real difference in the phospholipid composition of each muscle

extract rather than to normal variation in the analytical technique, since the differences were often statistically significant.

The mean phospholipid composition for each adaptational group was calculated separately. The results are shown in Table 2-5 where they are statistically compared. Of all the separated components compared only sphingomyelin proved to comprise significantly different proportions of the total phospholipid.

Effect of Thermal Acclimation upon the Cholesterol Content of Muscle

The cholesterol content of each total lipid extract from crayfish muscle was determined in triplicate using the method of Watson (1960). The total phosphorus content was determined as described in the 'Methods'. The yield of both cholesterol and phosphorus was expressed as uM per gram wet weight of muscle. The results for each extract are presented in Tables 2-3 and 2-4 for lipid extracts from 4°C and 25°C acclimated crayfish, respectively.

The yield of cholesterol from crayfish muscle varied between 1.32 and 3.67uM cholesterol/gram wet muscle weight, but averaged 3.06 $\pm$ 0.15 and 2.90 $\pm$ 0.55uM cholesterol/gram wet muscle weight for crayfish acclimated to 4°C and 25°C respectively. This difference was not statistically significant ($P > 0.7$, Table 2-5).

The yield of lipid phosphorus from crayfish muscle was found to vary widely between 3.87 and 10.98uM/gram wet muscle weight (see Tables 2-3 and 2-4). The cause of this variability is not known but may be associated with a variable water content of the original muscle, or perhaps with differential extraction efficiencies of phospholipid from different samples of muscle.

Table 2-2: Summary of the reactions to various spray reagents of the crayfish muscle phospholipid fractions and their tentative identifications

Methods: For the various spray reagents, see 'Materials and Methods'.

Legend:

* Blue reaction with the phosphate-specific spray reagent

** See Figure 2-4

Table 2-2: Summary of the reactions to various spray reagents of the crayfish muscle phospholipid fractions and their tentative identifications

Fraction No.**	Specific Spray Reagents					Cochromatography with -	Tentative Identity	Abbreviation
	Phosphate	Ninhydrin	Resorcinol	Dragendorff	Plasmalogen			
1	+ *	-	-	+	-	Sphingomylin	Sphingomyelin	Sph
2	+ *	+	-	-	+	Phosphatidyl Serine, Phosphatidyl Inositol	Serine and Inositol Phospholipids	PS + Pl
3	+ *	-	-	+	+	Phosphatidyl Choline	Choline Phospholipids	PC
4	+ *	+	-	-	+	Phosphatidyl Ethanolamine	Ethanolamine Phospholipids	PE
5	+ *	-	-	-	-	-	Phosphatidic Acid	PA
6	+ *	-	-	-	-	Cardiolipin	Cardiolipin	Cd.
7	+ *	-	-	-	-	-	Cerebroside (?)	Ce.
8	+ Red	-	-	-	-	Cholesterol	Cholesterol	Ch.
9	-	-	-	-	-	Neutral Lipids	Triglycerides, Fatty Acids, etc.	-
10	-	-	-	-	-	-	Fatty Acids	-

Legend: * Blue reaction with the phosphate-specific spray reagent (see Figure 2-4)

Table 2-3: The phospholipid composition, total phospholipid and cholesterol content of muscle lipids extracted from crayfish acclimated to $4^{o}C$

Methods: The total phospholipid and cholesterol content of each lipid extract was determined as described in 'Materials and Methods'. Values reported are the average of duplicate analyses and are uM Phosphorus or cholesterol/gm wet muscle weight. The phospholipid composition of each lipid extract was analysed in replicate. Values reported are the % of the total phosphorus recovered from the chromatoplate and are the mean $\pm$ S.E.M. of the replicate analyses.

Table 2-3

	Animal 1	Animal 2	Animal 3	Animal 4	$\bar{X}$	S.E.M.
Origin	0.76* (0.45-1.11)**	0.25 (0.20-0.90)	1.09 (0.92-1.27)	0.99 (0.80-1.19)	0.77	0.19
Sphingomyelin	5.24 (4.97-5.54)	4.99 (3.92-5.67)	4.26 (4.08-4.44)	4.60 (4.00-5.13)	4.77	0.22
Choline Phospholipids	51.90 (50.88-52.95)	54.42 (52.24-56.07)	56.65 (52.92-60.38)	53.40 (51.49-54.55)	54.09	1.00
Ethanolamine Phospholipids	28.78 (27.37-29.63)	24.90 (20.85-27.38)	22.07 (20.19-23.23)	29.05 (27.76-29.96)	26.20	1.67
Serine/Inositol Phospholipids	10.97 (10.64-11.10)	11.36 (10.62-11.27)	8.84 (8.67-9.00)	11.08 (9.96-11.67)	10.56	0.58
Phosphatidic Acid	0.42 (0.11-0.55)	0.61 (0.41-0.73)	0.86 (0.41-1.32)	0.10 (0.06-0.30)	0.50	0.16
Cardiolipin	1.37 (0.92-1.68)	1.45 (0.90-2.13)	1.03 (0.44-1.63)	0.34 (0.32-0.36)	1.05	0.25
Cerebrosides (?)	0.54 (0.47-0.70)	1.02 (0.40-1.92)	1.81 (1.48-2.15)	0.39 (0.30-0.46)	0.94	0.32
Neutral Lipids	0.00	1.02 (0.90-1.21)	0.92 (0.31-1.52)	0.58 (0.19-0.81)	0.84	0.13
Pi/Cholesterol Ratio *	1.95	2.22	3.23	-	2.47	0.39
uM Pi/gm wet muscle weight *	6.14	7.24	8.93	-	7.44	0.81
uM Cholesterol/ gm wet muscle weight *	3.15	3.27	2.76	-	3.06	0.15
n	4	4	2	3	-	-

n Number of phospholipid composition analyses

** range of values

- Not determined

* Mean of duplicate or triplicate analysis for each lipid extract

Table 2-4: The phospholipid composition, total phospholipid and cholesterol content of muscle lipids extracted from crayfish acclimated to 25°C

Methods: The total phospholipid and cholesterol content of each lipid extract was determined as described in 'Materials and Methods'. Values reported are the average of duplicate analyses and are uM Phosphorus or cholesterol/gm wet muscle weight. The phospholipid composition of each lipid extract was analysed in replicate. Values reported are the % of the total phosphorus recovered from the chromatoplate and are the mean $\pm$ S.E.M. of the replicate analyses.

Table 2-4

	Animal 1	Animal 2	Animal 3	Animal 4	$\bar{X}$	S.E.M.
Origin	0.32* (0.11-0.61)**	1.99	0.57 (0.25-0.85)	2.61 (2.31-2.79)	1.37	0.55
Sphingomyelin	5.99 (4.78-6.84)	8.47	5.41 (4.89-5.98)	6.98 (6.41-7.54)	6.71	0.67
Choline Phospholipids	54.63 (50.58-57.53)	53.26	58.60 (57.57-59.57)	53.39 (47.83-59.27)	54.97	1.25
Ethanolamine Phospholipids	24.28 (22.73-25.89)	22.63	23.84 (21.31-25.14)	20.65 (19.68-22.19)	22.85	0.81
Serine/Inositol Phospholipids	10.13 (9.35-10.70)	10.70	9.74 (8.09-11.18)	9.57 (9.02-10.37)	10.04	0.25
Phosphatidic Acid	0.79 (0.74-0.83)	0.47	0.31 (0.12-0.42)	0.81 (0.57-1.17)	0.60	0.12
Cardiolipin	1.07 (0.38-1.70)	0.88	0.58 (0.43-0.84)	2.54 (1.54-4.05)	1.26	0.44
Cerebrosides (?)	1.45 (0.76-2.27)	1.01	0.79 (0.57-0.92)	2.46 (1.32-3.46)	1.43	0.37
Neutral Lipids	1.97 (0.50-3.41)	2.35	0.24 (0.11-0.17)	3.28 (1.53-6.40)	1.96	0.63
Pi/Cholesterol Ratio *	1.87	3.06	2.19	2.92	2.51	0.29
uM Pi/gm wet muscle weight *	5.63	10.98	8.02	3.87	7.13	1.54
uM Cholesterol/gm wet muscle weight *	3.02	3.59	3.67	1.32	2.90	0.44
n	4	1	4	3	-	-

* Mean of duplicate or triplicate analysis for each lipid extract.

** range of values

n Number of phospholipid composition analyses

Table 2-5: Comparison of the average phospholipid composition, total phospholipid and cholesterol content of muscle lipid extracts from four crayfish acclimated to 4°C, and four crayfish acclimated to 25°C

Methods: Total phospholipid and cholesterol content of each extract was analysed in duplicate as described in 'Materials and Methods'. Values reported are the mean ± S.E.M. for four extracts from each acclimation group. The phospholipid composition of each lipid extract was analysed in replicate and the average phospholipid composition for each extract is reported in Tables 2-3 and 2-4. The values reported here are the mean ± S.E.M. composition of four crayfish muscle lipid extracts from each acclimation group.

Legend:

* - probability for (n-2) degrees of freedom.

** - values are % total phosphorus recovered from chromatoplate and are the mean ± S.E.M. for four lipid extracts from each acclimation group.

Table 2-5

Phospholipid Class	% Total phospho-Lipid 4°C adapted	% Total phospho-lipid 25°C adapted	n	t	P*
Sphingomyelin	4.77±0.22 **	6.71±0.67	8	2.75	0.02-0.05
Choline Phospholipids	54.09±1.00	54.97±1.25	8	0.55	0.06-0.70
Ethanolamine Phospholipids	26.20±1.67	22.85±0.81	8	1.80	0.1-0.2
Serine/Inositol Phospho-lipids	10.56±0.58	10.04±0.25	8	0.82	0.4-0.5
Phosphatidic Acid	0.50±0.16	0.60±0.12	8	0.50	0.6-0.7
Cardiolipin	1.05±0.25	1.26±0.44	8	0.41	0.6-0.7
Cerebrosides (?)	0.94±0.32	1.43±0.37	8	0.94	0.3-0.4
Neutral Lipids	0.84±0.13	1.96±0.63	8	1.74	0.1-0.2
Pi/Cholesterol Ratio	2.47±0.39	2.51±0.29	7	0.08	>0.9
uM Pi/gm wet muscle weight	7.44±0.81	7.13±1.54	7	0.18	0.8-0.9
uM Cholesterol/gm wet muscle weight	3.06±0.15	2.90±0.55	7	0.28	0.7-0.8

Again the difference between the average yield of lipid phosphorus from 4°C and 25°C acclimated crayfish was not statistically significant ($P > 0.8$, Table 2-5).

The molar phosphorus/cholesterol ratio is probably a more valid measure of the gross chemical composition of cellular membranes since it should not be affected by poor extraction efficiency or by muscle water content. Indeed, the variation in ratios between different lipid extracts with the same acclimation history is more limited (Tables 2-3 and 2-4). Nevertheless, the difference in the average phosphorus/cholesterol ratio for 4°C and 25°C acclimated crayfish was not statistically significant ($P > 0.9$, Table 2-5).

DISCUSSION

Crayfish muscle membrane phospholipids consist mainly of choline, ethanolamine and serine/inositol phosphoglycerides (approximately 55%, 26% and 10% respectively). This composition is very similar to that reported for the skeletal muscle and other tissues from a wide variety of vertebrate and invertebrate animals (Table 2-6). This is in accordance with the suggestion of Rouser, Simon and Kritchevsky (1969), that "among vertebrates, neither the relative proportions of different membranes of organs, nor the phospholipid composition of the individual types of membrane varies appreciably". Notable exceptions to this generalisation are the marine molluscs which contain appreciable quantities of ceramide – aminoethyl phosphonate.

The detailed phospholipid composition reported here agrees closely in most respects with the analysis of lobster abdominal muscle reported by Simon and Rouser (1969; see Table 2-6), although crayfish muscle possessed a slightly higher proportion of ethanolamine phosphoglyceride. There are, however, some

interesting considerations in this comparison.

Firstly, Simon and Rouser (1969) identified both serine and inositol phosphoglycerides as components of lobster muscle phospholipids. It is significant that the combined percentage of 11.1% for these phospholipids agrees closely with the value of 10.3% reported here for the PS/PI fraction. This suggests that, although the presence of inositol phosphoglyceride was not positively confirmed, crayfish muscle does possess it. The failure to separate serine and inositol phosphoglycerides by thin-layer chromatography is probably due to some differences in chromatographic technique.

Secondly, lysolecithin was not observed at any point during the chromatographic examination of crayfish muscle phospholipids. This is in contrast to the presence of 0.5 - 1.0% lysolecithin in lobster, abalone and scallop muscle phospholipids (Simon and Rouser, 1969), and the decapods Cyclograpsus sp. and Potamon sp. (De Koenig, 1970). The significance of lyso-derivatives in biological membranes is uncertain. They may represent intermediates in synthetic or degradative pathways, or may be the result of the degradation of lecithin, caused by post-mortem enzymatic action, or by autoxidation during the extraction, storage and chromatography of the lipid extract.

Thirdly, the variation in the muscle phospholipid composition between different crayfish, is of the same order experienced by Rouser, Simon and Kritchevsky (1969). Each lipid extract (except one) was analysed several times, in order to demonstrate that the difference between acclimation groups was probably due to biological variation in phospholipid composition.

The interpretation of the phospholipid composition of membranes is uncertain, mainly because little is known concerning the specific functions of the various phosphoglyceride classes within the membrane. The high proportion of the zwitterionic

choline - containing phospholipids (i.e., choline phosphoglycerides and sphingomyelin) in animal membranes suggest a general solvent role, whilst the presence of more limited quantities of the other phosphoglycerides indicates perhaps some more specific function. Recent reports of the reactivation of delipidated membrane-enzymes by specific phospholipid classes, supports this latter suggestion, even though most phospholipid classes have at some time been implicated in such phenomena (for review, see Coleman, 1973). In addition, the ethanolamine and serine phosphoglycerides are negatively charged at physiological pH's. This would impart an overall negative charge to the lipid membrane, which would be of obvious importance to its ionic interaction with proteins and ions.

Thermal acclimation clearly has no major effect upon the phospholipid class distribution of crayfish muscle membranes other than a slight elevation of the sphingomyelin content from $4.77 \pm 0.22\%$ to $6.71 \pm 0.67\%$ ($P < 0.05$, Table 2-5). This conclusio is in accordance with the conclusion by Roots (1968) that a "change in environmental temperature does not influence the amount of the major phospholipids found in goldfish brain". Although the phospholipid and cholesterol content of crayfish muscle varies widely, it was not possible to demonstrate any consistent differen between crayfish acclimated to $4^{\circ}C$ and those acclimated to $25^{\circ}C$. Similar results were reported in goldfish brain by Roots (1968), goldfish intestinal mucosa by Kemp and Smith (1970), Pseudomonas by Cullen, Phillips and Shipley (1971) and other goldfish tissues by Anderson (1970).

Table 2-6 The phospholipid composition of vertebrate and invertebrate tissues

Animal	INVERTEBRATES				VERTEBRATES							
	Crayfish*	Lobster	Cyclograp-sus sp.	Potamon sp.	Bovine	Human	Rat	Mouse	Frog	Rat	Rabbit	Pig
Tissue	Abdominal Muscle	Abdominal Muscle	Whole Animal	Whole Animal	Skeletal Muscle	Skeletal Muscle	Skeletal Muscle	Skeletal Muscle	Skeletal Muscle	Liver	Heart	Heart
Sphingomyelin	5.74	8.4	5	4	4.5	4.0	2.7	3.6	3.1	1.83	5.0	1.7
Lysolecithin	-	1.1	1	1	0.7	-	2.7	0.6	0.4	0.87	0.7	0.5
Choline Phospholipids	54.53	54.6	57	52	46.5	48.0	51.1	52.4	55.2	54.96	42.2	42.4
Serine Phospholipids	~~10.30~~	6.2	5	2	4.1	3.3	3.7	4.0	3.4	3.02	3.3	-
Inositol Phospholipids		4.9	4	4	5.6	8.8	8.9	6.7	5.5	8.81	3.6	4.5
Ethanolamine Phospholipids	26.55	23.6	22	27	26.6	26.4	22.2	25.8	29.0	25.32	30.9	30.6
Phosphatidic Acid	0.55	0.2	-	-	0.3	1.2	0.8	0.1	0.1	-	1.0	-
Cardiolipin	1.15	1.3	5	10	8.9	6.6	1.4	5.8	2.3	5.12	11.6	18.1
Others	2.58	tr	1	-	0.4	1.0	2.5	0.7	0.4	0.23	-	3.0
Reference	Present Work	Simon and Rouser (1969)	De Koenig (1970)		Simon and Rouser (1969)					Skipski et al (1964)	Owen (1966)	Comte, Gautheron, Peypoux Michel (1971)

* Average of 8 animals acclimated to both 4°C and 25°C. Table 2-5.

\- Absent

tr Present in trace amounts.

Chapter 3

THE EFFECTS OF THERMAL ACCLIMATION AND PHOTOPERIOD UPON THE FATTY ACID COMPOSITION OF MEMBRANE PHOSPHOLIPIDS

INTRODUCTION

Perhaps the most significant conceptual advance in recent membrane research has been a greater understanding of the physical state of the membrane. The elegant experiments of Frye and Edidin (Edidin, 1972) using fluorescent antibodies as membrane markers, and McConnell and his colleagues (McConnell, Devaux and Scandolla, 1972) using advanced spin-label techniques have demonstrated that at temperatures within the biological range, membranes may exist as highly dynamic systems, with a considerable degree of intermolecular motion and interactions. Indeed, so rapid is this motion that it has been estimated that a single phospholipid molecule in an E. coli bacterium could, through random molecular motion alone, travel from one end of the bacterium to the other, in the order of one second (McConnell, Devaux and Scandella, 1972).

As discussed in Chapter 2, the rate of passive permeability through a biological membrane depends, amongst other factors, upon the overall state of organisation of its component parts. The major barrier to the free diffusion of water-soluble solutes is the hydrocarbon core of the membrane and the more ordered and compact this hydrocarbon core is, then the more effective a barrier it is to the passage of solutes. The stability of this hydrophobic environment depends upon the hydrophobic interactions between the hydrocarbon chains of the phospholipids. The London-Van der Waals forces characteristically operate over very short distances, since the energy of interaction varies as the reciproca of D^6, where D is the distance between the interacting components. All of these interactions, therefore, will be highly dependent

upon the geometry of the molecules, which determine how closely they can pack. The closer the hydrocarbon chains can approach each other then the greater are the hydrophobic interactions and the greater are the co-operative retraining influences upon their molecular vibrations.

Any factor which leads to the disruption of the ordered state of the hydrocarbon environment will not only disrupt the membrane structure directly, but will also nullify the restraining influence of the hydrophobic interactions upon the molecular mobility. Both of these factors lead to a marked reduction in the overall stability of the hydrocarbon core, resulting in an increase in its permeability.

The effect of phospholipid headgroups and cholesterol upon the integrity of biological membranes has been discussed in Chapter 2. Another important factor in this respect is the nature of the hydrocarbon chains of the membrane phospholipids. Van Deenen and his colleagues, using monomolecular layers at an air-water interface, have demonstrated that the area occupied by a lecithin molecule depends very much upon its constituent fatty acids. With shorter saturated fatty acids there is a decrease in the hydrophobic interactions, resulting in a greater degree of chain mobility and a more expanded film. This also applies to fatty acids possessing cis-unsaturated bonds, since the kinked structure of the fatty acids impedes the close approach of neighbouring fatty acids and thereby denies them hydrophobic interactions (Demel, Van Deenen and Pethica, 1967).

It is to be expected therefore that the permeability of lipid bilayers would also depend upon the fatty acid constituents. This has been verified over the past 8 years, again by Van Deenen and his colleagues with a series of experiments performed on natural membranes, their derived 'liposomes' and on liposomes prepared from purified phospholipds.

De Gier, Mandersloot and Van Deenen (1968) prepared liposomes composed of 96 mole % synthetic lecithin (with different hydrocarbon constituents) and 4 mole % phosphatidic acid. All the liposomes proved to be almost ideal osmometers, and their initial swelling rates in isotonic solutions of glycerol, erythritol (De Gier, Mandersloot and Van Deenen, 1968) or glucose (Demel, Kinsky, Kinsky and Van Deenen, 1968) demonstrat that increasing the unsaturated fatty acid content of the lecithin component led to a marked increase in liposome permeability. For example, liposomes composed of synthetic (dilineoyl) phosphatidyl choline and phosphatidic acid were permeable to erythritol at 10°C, whereas liposomes composed of (dipalmitoyl) phosphatidyl choline and phosphatidic acid were permeable only above 40°C. (De Gier, Mandersloot and Van Deenen, 1968). The activation energy for the permation process appeared to be more characteristi of the permeant molecule, since it was approximately 21 Kcal. $mole^{-1}$. (87.9 $KJ.mole^{-1}$.) irrespective of the chemical composition of the hydrocarbon core of the membrane (De Gier, Mandersloot and Van Deenen, 1968; De Gier, Mandersloot, Hupkens, McElhaney and Van Beek, 1971).

These observations are supported by the observations of Klein, Moore and Smith (1971), that the rate of efflux of labelled neutral amino-acids from pre-loaded liposomes depended upon the degree of un-saturation of the lecithin used. Scarpa and De Gier (1971) have made similar observations with K^+ permeability, also in liposomes. The influence of variations in the hydrocarbon core are not limited to simple permeability phenomena, but may be reflected in more complex facilitated transport mechanisms and active membrane transport systems. For example, the valinomycin-induced leakage of $^{86}Rb^+$ from liposomes is strongly dependent upon the hydrocarbon core (De Gier, Haest, Van der Neut-Kok, Mandersloot and Van Deenen, 1972).

Additional evidence of the relationship between membrane permeability and phospholipid unsaturation in natural membranes has been provided by Wessels (quoted by De Gier, Haest, Van Der Neut-Kok, Mandersloot and Van Deenen, 1972). He noted a close correlation between the permeability to glycerol of erthrocytes from different mammalian species and an index of the unsaturation content of the erythrocyte membranes.

The observation by various workers that the permeation process is highly temperature dependent (see Haest, De Gier and Van Deenen, 1969; McElhaney, De Gier and Van Der Neut-Kok, 1973) has important implications for all poikilothermic organisms. A change in temperature from that normally experienced by an organism would result in marked effects upon the stability and passive permeability characteristics of its cellular membranes. A rise in temperature would increase the kinetic energy of the molecules, resulting in a less compact packing of the hydrocarbon chains. In addition, there would be a reduction of the London-Van der Waals forces which restrain the molecular motion of the chains in a co-operative fashion. These effects of temperature would be of particular relevance in tissues where the membrane permeability has a critical functional importance, such as muscle and nerve. Indeed, this phenomenon may serve to explain the breakdown in passive permeability properties of the sarcolemma during heat death in the freshwater crayfish as described by Bowler, Duncan, Gladwell and Davison (1973), and Gladwell (1973).

In a number of organisms, maintenance at a different environmental temperature is known to cause changes in membrane lipid composition (see Table 3-15). These changes are thought to compensate, either totally or partially, for the effect of temperature upon membrane functions, resulting in their preservation. It is possible that similar compensatory changes in membrane biochemistry could account for the differences in the

kinetics of heat death in the crayfish and the thermal stability of the sarcolemma that are caused by acclimation to different temperatures.

The present study was designed to determine whether a compensatory mechanism of this type exists in crayfish muscle, which may account for the increase in thermostability of the sarcolemma that occurs during thermal acclimation to a higher environmental temperature (Gladwell, 1973, Bowler, Gladwell and Duncan, 1973). Crayfish were acclimated to 4°C and 25°C and the fatty acid composition of the total phospholipid and the major purified phospholipids was determined using gas-liquid chromatography. Finally, the effects of photoperiod upon the fatty acid composition of the total phospholipids of crayfish acclimated to 4°C were studied.

MATERIALS AND METHODS

A. MATERIALS

1. Glassware. The source of all glassware used has been described in Chapter 2.

2. Animals. Crayfish (Austropotamobius pallipes) were obtained locally, and maintained in the laboratory as described in Chapter 2.

3. Chemicals. In addition to those chemicals detailed in Chapter 2, the following reagents were obtained from British Drug House Chemicals Ltd. (Poole, Dorset): 14% Boron-Trifluoride in Methanol; Hexane; Carbon disulphide; Silver Nitrate; and were 'Analar' grade. 10% polyethylene glycol adipate on Celite CAW (100-120 mesh) was obtained from Pye Unicam Ltd. (York Street, Cambridge). 'White Spot' nitrogen was supplied by the British

Oxygen Company Ltd. (Birtley, Co. Durham). Pure hydrogen and hydrocarbon-free air was supplied by B.O.C., Special Gases Division (Deer Park Road, London). Authentic Fatty Acid Standards were purchased from Sigma London Chemical Co. Ltd. (Kingston-upon-Thames), Supelco Ltd. and Applied Science Ltd. as detailed in Table 3-1.

B. METHODS

1. Treatment of glassware. Techniques for the cleaning of all glassware were described in Chapter 2.

2. Acclimation of Crayfish. Long Daylength Acclimation: Crayfish were acclimated to $4^{\circ}C \pm 0.5^{\circ}C$ or $25^{\circ}C \pm 0.1^{\circ}C$ both with an 18 hour-light daylength as described in Chapter 2. Short Daylength Acclimation: Crayfish were acclimated at $4^{\circ}C \pm 0.5^{\circ}C$ in a cabinet maintained in a $4^{\circ}C$ cold room. An 8 hour-light daylength was provided by miniature fluorescent lights controlled by a Veneret Mark IIA time switch.

3. Gas-Liquid Chromatography. Fatty-acids were analysed as their methyl esters on a Pye Unicam Series 104 model 24, Dual Column, Flame ionisation Gas Liquid Chomatograph.

(a) Preparation of Chromatography Columns. Glass columns fitted with glass-metal terminations were soaked overnight in 5N hydrochloric acid. The column was rinsed in distilled water followed by acetone. Finally, acetone was removed by passing nitrogen gas through the column at $105^{\circ}C$.

The stationary phase was introduced to the open end of the column through a glass funnel and

packed with nitrogen gas at 30-35 p.s.i. (2 bar). The packed columns were purged in the chromatograph oven at 225°C for 24 hours before use, with carrier gas passing through continuously at 10-20 ml/minute. Columns prepared by this technique have an optimal efficiency of 1,700-1,800 theoretical plates/metre (calculated for stearate methyl ester). Use of a 'Prespak' perspex reservoir (Phase Separations Ltd., Queensferry, Flintshire) to aid column packing, resulted in reduced column efficiencies of approximately 1,500-1,600 theoretica plates (for stearate methyl ester)/metre.

(b) Operating Specifications

(i) Column: 1500mm x 6mm o.d. (4 mm i.d.) glass with glass-metal termination (Pye-Unicam Ltd.)

(ii) Stationary phase: 10% Polyethylene glycol adipate.

(iii) Support: Acid washed Celite, 100-120 mesh.

(iv) Carrier Gas: 'White Spot' nitrogen. Flow rate 45 ml/min.

(v) Combustion Gases: Hydrogen 45 ml/minute
Hydrocarbon-free air 700-750 ml/minute
Hydrogen gas was further purified by passing it through a 190 mm cylinder of Type 13X Activated Molecular Sieve (Pye Unicam Ltd.)

(vi) Detector: Flame Ionisation:
Limits of detection under optional conditions 10^{-10} g/ml (see Figure 3-2)

(vii) Amplifier Sensitivity: Maximum 1 x 10^{-12} Amp
full scale deflectio
Minimum 5 x 10^{-7} Amps
f.s.d.
(manufacturers'
estimate)

(viii) Amplifier accuracy: Absolute $\pm$ 4% (manu-
facturers' estimate)
: Between two adjacent
positions of "Attenuato
switch, $\pm$ 1% (manufactu-
rers' estimate)

(ix) Jet Polarising Voltage: $\pm$ 170 V, stabilised
supply.
Negative with respect
to earth.

(x) Column temperature: 198 $\pm$ 0.5°C. Isothermal
Analysis.

(xi) Recorder: Servoscribe R.E.511.20 Potentio-
metric Recorder (A. Gallenkamp Ltd.
Chart speed - 120 mm/hr
F.S.D. - 10mV.
Response time - 1 sec

(xii) Injector Syringe: Hamilton 700 series 10ul
syringe (700L SN WG, with Kel-F
Guide)
$4\frac{1}{2}$ inch (115 mm) fixed needle with
17° point Style for septum
penetration.
Accuracy $\pm$ 1%
Reproducibility $\pm$ 1%
(Both manufacturers' estimates)

(c) Calibration of Carrier Gas flowrate

In common with all chromatographic processes, the flowrate of the eluant determines the speed of the analysis. The optional elution rate is necessarily a compromise between keeping the sample on the column long enough to ensure adequate separation of the mixture, and the peak-broadening effects of molecular diffusion. This latter process increases with analysis time and causes a decrease in peak resolution.

The most commonly used expression for the quality of a chromatographic column is the number of theoretical plates, which is a measure of the relative sharpness of a peak (Schupp, 1968)

$$n = 16\left(\frac{Rt}{W}\right)^2 \qquad \text{Equation } 3 - 1$$

where n - number of theoretical plates

Rt - is the retention time of the resolved solute

W - peak width at the base line cut by tangents of the slopes of the peak.

This equation is derived from distillation theory and latterly adapted for chromatography theory (Schupp, 1968). It depends upon the fundamental assumption that the factors contributing to peak spreading are random variables(e.g. molecul diffusion) and the peak describes a Gaussian distribution. The value of n, therefore, is an estimate of the variance of the peak.

A second measure of the quality of a column under stated operating conditions, is the resolution of components being separated. The resolution of two peaks may be defined by the expression

(Schupp, 1968):

$$\text{Resolution} = \frac{2\,\Delta Rt}{(W_1 + W_2)} \qquad \text{Equation 3-2}$$

where ΔRt is the difference between the retention time of the two components.

W_1 and W_2 are the peak width at the baseline cut by the tangents of the slopes of the peak.

If the Resolution $>$ 1 then the separation of the two components is at least 98% complete (Craske 197

In order to determine the carrier gas flow rate for optimal separation of components these two parameters were measured at various carrier gas flow rates between 30 and 100ml/minute. The results are illustrated graphically in Figure 3-1. Both parameters indicate a clear optimum carrier gas flow rate of 40-45ml/minute.

Although analysis time is reduced at higher flow rates, peak resolution and column efficiency fall off dramatically. Therefore a carrier gas flow rate of 45ml/minute was maintained for all subsequent analyses.

In accordance with the recommendations of Maggs (1966), the hydrogen flow rate was maintained equal to, or slightly in excess of, the carrier gas flow rate. Hydrocarbon-free air flowed at 700-750ml/minute. All flow rate measurements were performed with a 'bubble flowmeter' (Pye Unicam Ltd.) and stopwatch.

(d) Absorptivity of the Column

Detailed examination of peak shape over the entire concentration range confirmed that peaks were of a Gaussian form. There was no evidence of peak assymetry (for palmitate methyl ester) as

might be expected by adsorption of applied components, except when grossly overloaded. Furthermore, the calibration line discussed below (Figure 3-2) was linear even at low sample loads, indicating that loss of the sample during analysis for saturated methyl esters at least, was not a serious problem.

(e) Calibration of Ionisation Detector Response

(i) Linear range of the detector. The mass response characteristics of the ionisation detector was tested by injection of serial dilutions of a stock solution of 10mg palmitate methyl ester /ml carbon disulphide (Figure 3-2). The detector responded in direct proportion to the load of sample applied to the column over the range 10^{-9}g to 10^{-4}g palmitate methyl ester (Figure 3-2). The extrapolated limit of detection was 10^{-10}g palmitate methyl ester.

The mass-response characteristics of other saturated and unsaturated fatty acid methyl esters were not examined in detail since authentic standard for all naturally occurring fatty acids were not commercially available. Furthermore, Klein (personal communication), Ackman (1969) and Horning, Ahrens, Lipsky, Mattson, Mead, Turner and Goldwater (1964) have found that the response correction factors relative to palmitate for a wide variety of saturated and unsaturated fatty acid methyl ester lay within the limits of experimental error of the analytical technique. Hence, no advantage lay in taking relative response factors into account.

(ii) Reproducibility of Analysis. It was recognised at the outset that detailed quantitative analysis of a complex mixture of saturated and unsaturated fatty acids would result in sizeable errors. Therefore, each analysis of a sample of fatty acid methyl esters was performed in triplicate at least, and the final result was expressed as a mean $\pm$ S.E.M. of all analyses.

(iii) Load Limits of the column-detector system. Overloading of the column became apparent with samples greater than 10^{-4}g palmitate methyl ester, since the peak becomes markedly assymetic and the peak height deviated from the linear response discussed previously. Care was taken to ensure that all samples lay within the linear concentration range defined earlier.

(f) Injection Technique

The following technique was found to result in the complete and reproducible injection of the sample onto the column:

(i) Prior to use, the syringe was washed thoroughly in a variety of non-polar solvents. Periodically, the syringe plunger was withdrawn and wiped with a tissue soaked in hexane. Cleanliness of the syringe was checked at frequent intervals by injection of aliquots of carbon disulphide.

(ii) Carbon disulphide was taken up, expelled from the syringe to remove all air bubbles and to create an effective barrel-plunger seal.

(iii) A 1 ul 'slug' of carbon disulphide was taken up followed by another lul air bubble. The 'slug' served to wash any sample remaining in the barrel onto the column.

(iv) 8 ul of the sample to be analysed was taken up, followed by another 1 ul air bubble. (This latter bubble acted as dead space during the injection when boiling of the sample might otherwise cause loss of part of the sample onto the column before injection of the remainder.)

(v) The flexible needle was carefully introduced onto the column through a rubber septum. The contents were rapidly injected and the needle withdrawn.

(vi) The syringe was repeatedly cleaned with carbon disulphide solution.

4. Preparation of Fatty Acid Methyl Esters

(a) Extraction and Chromatography of Phospholipids

(i) Purified phospholipids. The abdominal muscle from variously acclimated crayfish were rapidly dissected out and the lipids extracted as described in Chapter 2. Phospholipids were fractionated on silica gel H using 2-dimensional TLC and visualise with Rhodamine 6G (Parker and Peterson, 1965) also as described in Chapter 2. The spots were scraped off the chromatoplate using a clean glass microscope slide, and taken up by suction into an elution trap (Goldrich and Hirsh, 1963). The phospholipids were eluted with a 1 ml portion of chloroform-methanol (2:1) containing 0.005% BHT followed by two 0.5ml portions.

(ii) Total phospholipid fraction. Contaminating cholestrol and neutral lipids were separated from the phospholipid fraction by thin layer chromatography using petroleum ether: Diethyl ether: Glacial acetic acid (70:30:2, v/v/v) as the developing solvent. The phospholipids remained at the

origin and were scraped off the chromatoplate and eluted as described above. Isolated phospholipids were stored under nitrogen in sealed glass ampoules at -20°C until required (up to 2 days).

(b) Interesterification Procedures

Isolated phospholipids were dried down in a glass ampoule under pure dry nitrogen and the hydrocarbon chains interesterified according to the procedure of Morrison and Smith (1964). 0.5ml 14% boron trifluoride in methanol was added to the dried phospholipids. The phospholipids were dissolved in the reagent, the ampoule was flushed with nitrogen and then sealed in a flame (Klopfenstein, 1971). The ampoule was incubated at 100°C for exactly 10 minutes. After cooling 0.5 ml distilled water and 1 ml of hexane were added and the fatty acid methyl esters were extracted into the hexane by creating an emulsion of the 2-phase system. The hexane and aqueous phases were separated by centrifugation at 1000g for 10 minutes in a Mistral 2L centrifuge (M.S.E. Ltd.). The upper hexane layer was removed using a Pasteur pipette and stored under nitrogen in sealed glass ampoules at -20°C until required for analysis.

(c) Structural Notation of Fatty Acids

The shorthand system used here for designating the structure of particular fatty acids is that of Ackman (1969) which is based upon the notation

$$n : x \quad \omega y$$

where n - is the total number of carbon atoms in the fatty acid chain (i.e., chain length)

x - is the number of olefinic double bonds in the carbon chain (i.e., degree of unsaturation)

y - is the number of carbon atoms from the centre of the double bond furthest removed from the carboxyl end group, to and including the terminal methyl group (i.e., end carbon chain).

This system has the advantage of indicating the structural relationships of biosynthetic importance, and also the structural parameters that define the GLC characteristics.

It is assumed that all olefinic bonds in unsaturated fatty acids are 'cis' in structure and are methylene interrupted although certain non-methylene interrupted isomers have recently been reported (Ackman and Hooper 1973). In this study all retention times are reported relative to methyl palmitate (16:0), since this compensates for day to day variations in operating conditions that result in a variability in the absolute retention time. The retention time was measured from the rising portion of the solvent front.

5. Identification of Peaks

(a) Peaks were tentatively identified by comparison of retention times with authentic samples obtained from commercial sources (Table 3-1).

(b) In those cases where authentic samples were not available peaks were tentatively identified using the 'semilog procedure' of Ackman (1963a). The relative retention times of homologs are known to be related in a logarithmic fashion to the number of carbon atoms present. This correlation depends solely upon the fatty acid chain length, the number of unsaturated bonds and the length of the end

carbon chain (for definition see section 4(c) in this chapter). Ackman (1963a) has shown that all such lines are parallel to the line for monosaturate methyl esters.

Thus, for methyl esters of normal methylene-interrupted polyunsaturated fatty acids, a straight line drawn through any point, parallel to the line established for monounsaturated fatty acids joins all points representing homologous fatty acids (i.e., with the same number of olefinic bonds and end carbon chain, but with different numbers of carbon atoms in the chain). With packed columns of the type used in this analysis separation of monoenoic isomers is not achieved. Thus, for plotting purposes, all monounsaturated fatty acids are assumed to be w9, although this may not be the case (Addison, Ackman and Hingley, 1968, 1972).

The data of Ackman (1963a), Burchfield and Storrs (1962) and that obtained in the present study using authentic fatty acids (Table 3-2), were plotted in this way. The RRt of fatty acid methyl esters that were either unavailable or not given in the literature were predicted by interpolation of these parallel lines.

(iii) The 'Separation Factor' procedure of Ackman (1963a was used to predict the RRt of fatty acid methyl esters that were not obtained by other procedures. The separation factor of two components of a homologous series, is the ratio of their relative retention times, and has a characteristic value for particular combinations of fatty acids. These can be used to predict the degree of unsaturation and position of

the double bonds of unknown fatty acid methyl esters (e.g., Ackman, 1963b, 1969; Johnson and Davenport, 1971). Ackman (1969) has summarised the different types of separation factor, and discusses their applicability to the problem of peak identification.

(iv) As a check on the identity of the major fatty acids present in crayfish muscle phospholipids, the derive fatty acid methyl esters of purified choline phospho glycerides were fractionated according to their degree of unsaturation by 'Argentation' thin-layer chromatography.

Choline phosphoglycerides were initially purified on 0.5mm silica gel H chromatoplates using the 2-dimensional system described in Chapter 2. The spot was taken up into an 'elution trap' and the phospholipids were eluted with three 1ml portions of chloroform/methanol (2:1) containing 0.005% BHT. The fatty acid methyl esters were prepared (see section 4b of this Chapter) and then applied to a TLC chromatoplate with a 250um layer of silica gel H containing 10% (w/w) $AgNO_3$. The chromatoplate was developed with petroleum ether (40-60°C, b.p.)/ diethyl ether/glacial acetic acid (70 : 30 : 2, v/v/v). The fractions were subsequently located using the Rhodamine 6G reagent (Chapter 2) and the methyl esters were eluted from each spot with hexane, and dried down for gas-liquid analysis. This procedure separates methyl esters according to their degree of unsaturation, and enables peaks that usually overlap to be examined separately.

(v) The results of these various procedures for the prediction of RRt of unavailable fatty acid methyl esters are summarised in Table 3-3.

6. Quantification of Peaks

It has been shown previously that the magnitude of ionisation detector response is linearly related to the load of methyl ester applied. It has also been experimentally verified in other laboratories (Maggs 1966; Horning, Ahrens, Lipsky, Mattson, Mead, Turner and Goldwater, 1963; Johnson and Davenport, 1971) that, for the different components of a mixture, the measurement of peak area gives a direct estimate of the weight of C_8 and higher fatty acids applied to the column. Furthermore there is no detectable difference in the mass response for long chain polyunsaturated fatty acids when compared with saturated compounds. Hence the major problem in quantifying chromatograms is the measurement of peak area.

The relative areas of each peak was estimated using the method of Carrol (1961) (see Section 3(d)). Here it is assumed that each peak describes a Gaussian curve and that the area is a product of the peak height and its standard deviation (Schupp 1968)

$$A = 2.507\ h.\ d. \qquad \text{Equation 3-4}$$

where A - area of the peak

h - maximum peak height

d - standard deviation of the peak

The standard deviation is usually estimated by measuring the peak width at half height $\left(W\frac{h}{2}\right)$ when

$$W\frac{h}{2} = 2.354\ d \qquad \text{Equation 3-5}$$

Thus $$A = \frac{2.507}{2.354}\ h.\ W\frac{h}{2}. \qquad \text{Equation 3-6}$$

However, this technique is unsatisfactory because errors in measurement of peak width become large with sharp peaks that elute rapidly. Since the standard deviation (d) of a peak is linearly related to its retention time (Carrol 1961) it becomes permissible to replace the peak width at half-height with the uncorrected retention time (Rt) in Equation 3.

$$\text{Hence } A = h \times Rt \qquad \text{Equation 3-7}$$

The weight-per cent. fatty acid composition of a mixture of fatty acids was therefore determined by estimating the relative areas of all component peaks. The areas of all peaks were summed and this total was assumed to represent the whole sample (i.e. 100%). The weight-per cent. of the n^{th} component was then given by -

$$Wt\%\ n = \frac{An}{\Sigma A} \cdot 100 \qquad \text{Equation 3-8}$$

where An - relative area of peak associated with compound n.

ΣA - Sum of relative peak areas in mixture.

Although the reproducibility of this technique is superior to others, Dijkstra (1961) has criticised it for samples containing compounds with very different RRt, since the relationship between peak width and Rt is only approximate. Nevertheless, the errors in the alternative methods make this approximation unimportant. However, it should be remembered that in calculating percentage composition, an error in measurement of one peak will result in altered values for all other components. Presentation of results to two decimal places is solely to allow the small propositions of various components to be shown. It does not imply accuracy at that level.

RESULTS

A. Identification of Fatty Acids of Purified Choline Phospholipid

(i) Limitations of GLC Indentification Procedures

It should be remembered that the comparison of relative retention times (RRt) of an unidentified chromatographic peak with the RRt of authentic compounds, or with the RRt predicted by various procedures, provides at best only a tentative identification of the unknown compound. This is because several quite distinct chemical compounds may exhibit similar chromatographic characteristics.

For the positive identification of each component, other analytical procedures should be employed, such as infra-red and mass spectrometry. In view of the complexity of such a complete analysis and bearing such limitations in mind, it was assumed that each peak does indeed represent a fatty acid methyl ester. It was further assumed that the structure of each component of a chromatographic analysis could be adequately described using the procedures described in 'Materials and Methods'

(ii) Purification and Fractionation of Lecithin Fatty Acids

The fatty acids present in a purified crayfish phospholipid were studied in detail by gas-liquid chromatographic (GLC) analysis following fractionation of the methyl esters by 'argentation' chromatography as described in the 'Materials and Methods'.
Five fractions were visualised on the chromatoplate under short wavelength UV light following 'argentation' chromatography, and these were numbered consecutively from the solvent front to the origin. Thus fraction I had an Rf of approximately 0.8, relative to the solvent front, whereas fraction V was almost contiguous with the origin.

The gas-liquid chromatogram for each fraction is illustrated in Figure 3-4. The relative retention times (1.00 = 16:0) for each chromatographic peak was compared with those values given in Table 3-3, and thereby 'tentatively' identified. The relevant data is presente in Table 3-4, where it can be seen that there is good agreement between the observed RRt for each unknown compound, its expected behaviour during 'argentation' chromatography, and the RRt of the corresponding authentic or predicted methyl ester. (It is emphasised that for illustrative purposes, Figure 3-4 is composed of chromatograms at various detector sensitivities and the peak areas illustrated therefore bear little or no relation to the actual composition of the mixture).

Fraction I consists entirely of saturated and monoenoic fatty and methyl esters (Table 3-4). The majo components of the mixture were 16:0, 16:1w9, 18:0 and 18:1w9. BHT was also present in this fraction (RRt = 0.45). The peaks eluting before 16:0 were

ignored during the quantitative and qualitative analysis, mainly because impurities and the antioxidant BHT obscures the chromatogram in this region. However, analyses in the absence of BHT indicated that 14:0, with which it overlaps was a very minor component (<0.2%) of the total mixture. A major peak (RRt = 0.860) was infrequently observed. This was ascribed to an impurity since its RRt did not correspond to any known or predicted fatty acid, and its highly variable magnitude. The peak with RRt = 1.55 may be either 17:1w9 or iso16:0 and it is not possible to decide between them.

Fraction II consisted of mono- and dienoic methyl esters (Table 3-4, Figure 3-4). In each case the correspondence between the observed RRt and the predicted RRt was close (Table 3-4). 18:2w6 was the only major component. 22:9 w9 could be observed only at high detector sensitivity.

Fraction III consisted mainly of trienoicmethyl esters. Although a large number of isomers were identified, only 18:3w3 was present in large amounts. 20:3w3 was positively identified as a minor component with RRt = 5.71.

Fraction IV consisted mainly of tetraenoic methyl esters, with 20:4 w6 comprising the major fraction. A minor peak corresponding to 20:1w9 was also observed.

Fraction V consisted mainly of pentaenoic and hexaenoic methyl esters (Table 3-4, Figure 3-4). 20:5w3 was present in large amounts, whilst 22:4w9, 20:5w6, 22:5w3 and 22:6w3 were present in smaller quantities. 22:5w6 and 18:4w3 were trace components.

Each TLC fraction was often contaminated by methyl esters from adjacent fractions of the $AgNO_3$ thin-layer chromatograms, and this complicated the identification procedure. For example, both fraction I and II contained peaks corresponding to 16:1w9, and to 18:1w9 (Table 3-4). Also fractions I and III contained a very minor peak (not illustrated in Figure 3-4) that corresponded well with the predicted RRt of 20:1w9. This was not such a problem for fractions II, IV and V. Clearly, the chromatographic procedure did not ensure complete fractionation between unsaturation classes. This may have been caused by overloading the TLC plate with sample.

However, this procedure did permit a more detailed analysis of the mixture of methyl esters than would otherwise have been the case. Thus it has proved possible to separate 20:3w3 (RRt = 5.71, fraction III) from 20:4w6 (RRt = 5.51, fraction IV), which otherwise would completely overlap even though their retention times are somewhat different (Ackman, 1969, p.365). In other cases, the distinction between the RRt of two fatty acids present in different TLC fractions is not great and the possibility remains that they are the same compound. Thus 18:3w3 (RRt = 3.13, Fraction III) and 18:4w6 (RRt = 3.07, Fraction IV) have very similar retention times, and the major evidence for their separate identity is their appearance in different TLC fractions. 20:4w3 (RRt = 6.40, Fraction IV) and 20:5w6 (RRt = 6.12, Fraction V) present a similar problem Therefore in those cases where the RRt's are significantl different (i.e., RRt $\gg$ 0.20), it is concluded that the components have different chemical structures.

It was concluded that phosphatidyl choline contains mainly 16:0, 16:1w9, 18:0, 18:1w9, 18:2w6, 20:4w6, 20:5w3, 22:5w3 and 22:6w3. A large number of other components were observed in minor or trace amounts.

B. The Fatty Acid Composition of Purified Total Phospholipids from Crayfish Muscle

(i) Treatment of Crayfish

Freshly caught crayfish were maintained for 1 week at $15\pm0.5^{\circ}C$ under 18hr-light daylength conditions. All crayfish were in the inter-moult condition. The crayfis were divided into two groups without bias to sex or size of each animal. Each group was acclimated either to $4\pm0.5^{\circ}C$ or to $25\pm0.1^{\circ}C$ for three weeks, both under 18hr-light photoperiod conditions.

(ii) Extraction and Identification of Fatty Acids

The phospholipids were extracted from the abdominal muscle of ten crayfish from each acclimation group, as described in Chapter 2. The total phospholipid fraction was purified by one-dimensional thin layer chromatography and their fatty acids prepared for GLC analysis as described in 'Materials and Methods' of this chapter. The identity of each chromatographic peak was tentatively established as in the previous section and the quantitative fatty acid composition of each phospholipid extract estimated using the method of Carroll (1961).

A typical gas-liquid chromatogram for the fatty acids of a total phospholipid fraction is presented in Figure 3-5. The RRt (16:0) for each chromatographic peak, and the RRt (16:0) for the associated authentic methyl esters (or predicted RRt (16:0) for unavailable methyl esters) are presented in Table 3-5 for comparison. The types of fatty acid identified in these total

phospholipid fractions were very similar to those established for purified choline phospholipids after 'argentation' chromatography. However, many of the minor or trace components identified in the latter study were totally obscured by the major components of the fatty acid mixture and therefore could not be included in the quantitative analysis.

Several peaks present in this analysis of purified phospholipid fatty acids were not observed in the previous study. These were tentatively identified from their RRt (16:0) values as 16:4w6 (RRt = 1.63), 17:2w4 (RRt 1.72), 18:4w3 (RRt = 3.43), 22:2w6 (RRt = 8.24) and 22:5w9 (RRt = 10.48). The latter three fatty acids were all trace components within the mixture of fatty acids and were of minor quantitative importance.

(iii) Quantitative Analysis

The total phospholipid fraction of each muscle lipid extract was purified and their fatty acids analysed five times separately. The results were averaged and are presented in Table 3-5 and illustrated in Figure 3-6.

The fatty acids of the total phospholipid fraction from both acclimation groups were composed mainly of four components, each comprising 10% or more of the total weight of fatty acid (Figure 3-6). These were 16:0, 18:1w9, 20:4w6 (+20:3w3) and 20:5w6. The latter two fatty acid fractions together comprised over 40% of the total weight.

Nine other components were present in quantities of 1-10%, principally 16:1w9, 19:0, 18:2w6 and 22:6w3. All other components were present in relative quantities below 1%.

(iv) Effects of Thermal Acclimation upon fatty acid composition

This analysis is based upon the comparison of phospholipid extracts from the abdominal muscle of ten crayfish of roughly equal size, from each acclimation group. It therefore represents the approximate average fatty acid composition of those ten animals.

Although the same general pattern of fatty acid composition is evident in the phospholipid fraction of both acclimation groups, there are some conspicuous differences in the proportions of the various components of the mixture (Figure 3-6). The extract from 4°C acclimated crayfish had reduced levels of the saturated fatty acids 16:0 and 18:0 and the polyunsaturate 22:6w3 when compared to the corresponding extract from 25°C acclimated animals. The polyunsaturated fatty acids 20:4w6 (+ 20:3w6) and 20:5w3 composed slightly higher proportions of the phospholipid fraction from 4°C acclimated crayfish muscle.

(v) Changes in Carbon Chain Length

When the data was grouped according to carbon chain length (Table 3-6a, and Figure 3-7a) it became apparent that there was a smaller proportion of C16 (difference of 1.37%, $P < 0.01$) and C22 (difference of 1.45%, $P < 0.001$) of fatty acids in the phospholipid fraction from the muscle of 4°C acclimated crayfish. This was compensated by an increase in the C20 component in this acclimation group (difference of 2.59%, $P < 0.001$).

(vi) Changes in Fatty Acid Unsaturation

By grouping the data according to the number of olefinic bonds present in the hydrocarbon chain (Table 3-6b and Figure 3-8a), it became clear that the phospholipid fraction from 4°C acclimated crayfish contained

a greater proportion of monoenoic (difference of 1.37%, P = 0.001-0.01), dienoic (difference of 1.49%, P<0.001), trienoic (difference of 0.25%, P = 0.02-0.05) and pentaenoic fatty acids (difference of 1.65%, P<0.001) than th phospholipids from 25°C acclimated crayfish.

The saturated (difference of 4.00%, P<0.001) and the hexaenoic components (difference of 1.15%, P<0.001) however, formed a smaller proportion of the total fatty acid weight in the former acclimation group.

The differences in fatty acid composition described above are all statistically significant and therefore demonstrate that the total phospholipid fraction from the 4°C acclimated crayfish had a slightly greater unsaturated fatty acid composition (i.e., 4.00% greater).

It has been demonstrated previously that over 50% of the total phospholipid of crayfish muscle is made up of choline phospholipids (Chapter 2). It was to be expected, therefore, that the fatty acid composition of the total phospholipid fraction would be dominated by the fatty acid composition of the choline phospholipids. Thus any differences in the fatty acid composition of other types of phospholipid caused by thermal acclimation would not be readily manifest in an analysis of the total phospholipid fatty acid composition. It was therefore decided to analyse the fatty acid composition of the major phospholipid fractions, the choline phospholipids (PC), the ethanolamine phospholipids (PE), and the serine/inositol phospholipids (PS + PI).

These phospholipid classes were fractionated by two-dimensional thin layer chomratography as described in 'Materials and Methods'. Each phospholipid class was eluted from the silica gel and its constituent fatty

acids analysed by GLC, also as described in 'Materials and Methods'. Analysis of each muscle phospholipid extract was performed in replicate, and the final result expressed as an average $\pm$ S.E.M. of 3, 4 or 5 separate analyses of the same extract.

C. The Fatty Acid Composition of the Choline Phospholipids

A typical gas-liquid chromatogram of the fatty acids of the choline phospholipids is presented in Figure 3-9.

As expected the basic pattern of the fatty acids of choline phospholipids was similar to the pattern described for the total phospholipid fraction (compare Figures 3-10 and 3-6). Thus 16:0, 18:1w9, 20:4w6 and 20:5w3 were the dominant fractions, comprising approximately 70% of the total weight of the fatty acid mixture (Figure 3-10 and Table 3-7). The major difference from the fatty acid composition of the total phospholipid fraction, was an increase in the proportion of 16:0, 16:1w9 and 18:1w9, with a corresponding decrease in the proportion of 20:4w6 (+20:3w3), 20:5w3 and 22:6w3 fatty acids.

Acclimation of crayfish to different environmental temperatures had a small effect upon the fatty acid composition of the choline phospholipids.

(i) Changes in Carbon Chain Length

There were no statistically significant differences in the proportion of fatty acids of each carbon chain length (Table 3-8a and Figure 3-7b).

(ii) Changes in Fatty Acid Unsaturation

Choline phospholipids from the abdominal muscle of the crayfish acclimated to $4^{\circ}C$ had a significantly higher proportion of dienoic (difference of 2.76%, $P < 0.001$) and trienoic fatty acids (difference of 0.44%, $P < 0.001$) (see Table 3-8b and Figure 3-8b) than the

corresponding fraction extracted from the muscle of 25°C acclimated crayfish.

In addition the former extract possessed a significantly lower proportion of the saturated fatty acids (difference of 4.09%, P = 0.001 - 0.1). The tetraenoic, pentaenoic and hexaenoic fractions comprised almost identical proportions of the total fatty acid weight of choline phospholipids in both extracts (Table 3-8b).

D. The Fatty Acid Composition of the Ethanolamine Phospholipids

The ethanolamine phospholipids of crayfish abdominal muscle possessed a very different fatty acid composition compared to the choline phospholipids (Figure 3-12 and Table 3-9). The saturated fatty acids 16:0 and 18:0, 18:1w9 comprised a smaller proportion of the total fatty weight of the ethanolamine phospholipids whilst the poly-unsaturated fatty acids together comprised over 60% of the mixture. 22:6w3 was present as a major fraction between 8-10% (Table 3-9).

Acclimation of crayfish to 4°C or to 25°C appears to have had marked effects upon the fatty acid composition of the ethanolamine phospholipids (Figure 3-12).

(i) Changes in carbon chain length

Ethanolamine phospholipids from the muscle of 4°C acclimated crayfish contained less C16 (a difference of 2.69 %, P = 0.001 - 0.01) and C22 fatty acids (difference of 3.30%, P = 0.001 - 0.01) than ethanolamine phospholipids from the muscle of 25°C acclimated crayfish (Table 3-10a, Figure 3-7c). Phosphatidyl ethanolamine from the former group also possessed significantly more C20 fatty acids (a difference of 3.92%, P = 0.001 - 0.01) than phosphatidyl ethanolamine from the latter group.

Phosphatidyl ethanolamine from both sources contained almost identical proportions of C18 fatty acids.

(ii) Changes in fatty acid unsaturation

Phosphatidyl ethanolamine from 4°C acclimated crayfish possessed somewhat less saturated fatty acids (a difference of 4.26%, $P<0.001$), and increased proportions of monoenoic (a difference of 3.09%, P = 0.001 - 0.01) tetraenoic (a difference of 1.04%, P = 0.001 - 0.01), and pentaenoic fatty acids (a difference of 3.12%, P = 0.01 - 0.02) compared to phosphatidyl ethanolamine from the abdominal muscle of 25°C acclimated crayfish (Table 3-10b, Figure 3-8c). It was interesting that phosphatidyl ethanolamine from the muscle of 4°C acclimated crayfish contained a somewhat smaller proportion of hexaenoic fatty acids (difference of 3.16%, $P<0.001$) than PE from 25°C acclimated crayfish.

Thus acclimation of crayfish to lower environmental temperatures has resulted in an overall increase in the unsaturation of phosphatidyl ethanolamine fatty acids. This was caused mainly by increases in the proportions of 17:1w9, 20:4w6 and 20:5w3. 22:6w3 is somewhat paradoxical in this respect since PE from the muscle of 4°C acclimated crayfish contained over 3% less than PE from the muscle of 25°C acclimated crayfish.

E. The Fatty Acid Composition of Serine and Inositol Phospholipids

The serine/inositol phospholipid fraction from crayfish muscle possessed a radically different fatty acid composition from the previously described phospholipid fractions, although the same types of fatty acid were identified. A typical gas-liquid chromatogram of PS + PI fatty acids is illustrated in Figure 3-13, and the results of a quantitative analysis of the fatty acid composition are presented in Table 3-11 and Figure 3-14.

Thus 16:0 comprised only 4% of the total and 16:1w9 was 1% or less. 18:0 became the dominant C18 fatty acid (19-25% approximately) and 18:1w9 became a minor component with only 5-7% approximately. 18:2w6 and 18:4w3 were both approximately 0.5%. The polyunsaturates 20:4w6 and 20:5w3 were present in large amounts (approximately 15-19% and 33-37% respectively). 22:6w3, however, was present only as a minor component of less than 2%.

Acclimation of crayfish either to 4°C or to 25°C appears to have caused marked changes in the fatty acid composition of this phospholipid fraction (Figure 3-14). The fraction from 4°C acclimated crayfish has reduced proportions of 18:0 and 22:6w3 when compared to the same fraction from 4°C acclimated crayfish. The former acclimation group also possessed increased proportions of 18:1w9, 20:4w6 and 20:5w3.

(i) Changes in carbon chain length

There were statistically significant differences in the proportion of C18 and C20 fatty acids in phosphatidylserine and phosphotidylinositol (PS + PI) from the abdominal muscle of 4°C and 25°C acclimated crayfish (Table 3-12a, Figure 3-7d). Thus this fraction from the former group of animals contained much less C18 fatty acids (difference of 5.42%, $P = 0.001\text{-}0.01$). This was compensated for by a marked increase in the proportion of C20 fatty acids (difference of 8.24%, $P < 0.001$) in the PS + PI fraction from 4°C acclimated crayfish compared to the corresponding fraction from 25°C acclimated crayfish.

(ii) Changes in fatty acid unsaturation

Statistically significant differences occurred in the saturated monoenoic, tetraenoic and pentaenoic unsaturation groups (Table 3-12b, Figure 3-8d). PS + PI

from the muscles of 4°C acclimated crayfish possessed a smaller proportion of saturated fatty acids than PS + PI from the muscle of 25°C acclimated crayfish (i.e., from 31.91% to 24.84%, see Table 3-12b and Figure 3-8d). Similarly hexaenoic fatty acids were slightly reduced from 1.93% to 1.37%, but this difference was just outside the limits of significance.

PS + PI from the muscle of 4°C acclimated crayfish contained larger tetraenoic (difference of 4.45%, P = 0.001-0.01) and pentaenoic fractions (difference of 3.12%, P = 0.01 - 0.02) than the corresponding fraction from 25°C acclimated crayfish.

The general effect of thermal acclimation upon the fatty acid composition of muscle PS + PI appears tocause a marked increase in the overall unsaturation of the phospholipid. This is mediated by reductions in the proportion of 18:0 and increases in the proportion of 18:1w9, 20:4w6 and 20:543.

F. <u>The Effect of Photoperiod upon the Phospholipid Fatty Acids of Crayfish Muscle</u>

Three crayfish were kept at 4±0.5°C under 8 hr-light photoperiod conditions for 21 days. The abdominal muscle from the animals was dissected out, and the total lipid was extracted in the usual manner. The phospholipids of five aliquots of this extract were separately purified by one-dimensional chromatography as described in 'Materials and Methods', and their constituent fatty acids analysed by GLC.

The analytical results for each phospholipd sample of the lipid extract were averaged. The average (± S.E.M.) fatty acid composition for the total phospholipid fraction from crayfish muscle is presented in Table 3-13.

The results for the 4°C, 8 hour daylength acclimated crayfish and the previously analysed total lipid fractions are illustrated in Figure 3-15 for comparison.

The same types of fatty acids were identified in the purified phospholipid fraction from all three muscle extracts except for 16:4w6 (RRt = 1.63) which was identified only in the extract from 25°C, 18 hr daylength, acclimated crayfish, 17:2w4 (RRt = 1.72), which was present only in the extract from 4°C, 18 hr daylength acclimated crayfish, and 20:2w9 (RRt 4.23), a trace amount of which was identified in the extract from 4°C, 8 hr daylength acclimated crayfish.

The purified total phospholipid fraction from the crayfish maintained at 4°C with 8 hr daylength possessed a fatty acid pattern that was broadly similar to those described previously for the other total phospholipid fractions. Thus the major fractions were 16:0, 18:1w9, 20:4w6 and 20:5w3. However, there were differences in the details of the fatty acid composition of the three extracts. For example, the 4°C, 8 hr daylength acclimated crayfish possessed less 16:0 than the other phospholipid fractions, and a great deal more 20:4w6 and 20:5w3.

The following description is based upon a comparison of the grouped data of the fatty acid composition of the extracts from both 4°C acclimated groups of crayfish. It is thought that this comparison would give information concerning the effects of changed photoperiod conditions only upon the biochemical composition of crayfish muscle. Both sets of data are characterised by small variations between the replicate analyses.

(i) Comparison of Carbon Chain Length (Table 3-14a, Figure 3-16a)

The phospholipids of short daylength-acclimated crayfish muscle possessed a smaller proportion of C16 fatty acids (difference of 2.29%, $P < 0.001$), caused by decreases in both 16:0 and 16:1w9 fatty acids. Similarly, there is a significant decrease in the C18 fatty acids (difference of 3.16%, $P < 0.001$, caused by a reduction in the proportion of 18:0, 18:1w9 and 18:2w6.

This is compensated by a significant and marked increase in the C20 fatty acids (difference of 5.44%, $P < 0.001$). The C22 fatty acids form the same proportion of the total in both phospholipid extracts.

(ii) Comparison of fatty acid unsaturation (Table 3-14b, Figure 3-16b)

The total purified phospholipid fraction from 4°C 8 hr photoperiod acclimated crayfish possessed a significantly smaller proportion of saturated (difference of 1.30%, $P = 0.001\text{-}0.01$), monoene (difference of 1.67%, $P = 0.001\text{-}0.01$), diene (difference of 1.04%, $P < 0.001$) and triene fatty acids (difference of 0.55%, $P < 0.001$). All of these differences are small, but none the less significant.

They are compensated for by marked increases in the proportion of tetraene (difference of 3.69%, $P < 0.001$) and pentaene fatty acid fractions (difference of 1.92%, $P < 0.001$). The hexaene fatty acids form the same proportion of the total in both extracts.

DISCUSSION

An interesting feature of these results is the clear distinction between the fatty acid composition of the individual phospholipid fractions. For example, choline phosphoglycerides possessed a large proportion of 16:0 (17 - 19%) whereas the ethanolamine and serine/inositol phosphoglycerides possessed small proportions (3-6%). Also the ethanolamine phosphoglyceride fraction alone possessed large amounts of 22:6w3, while the serine/inositol phosphoglycerides in contrast to the other phosphoglyceride fractions, possessed more 18.0 than 18:1w9. It is not easy to see why these differences exist. It may simply be that the synthetic pathways of the various phospholipid classes are different and that the variation in fatty acid composition has no special functional significance. On the other hand it could be considered to infer some functional division of labour between the phospholipid classes.

In agreement with the results of Wolfe, Rao and Cornwell (1968), crayfish muscle phospholipids, particularly the acidic oomponents, were relatively rich in long chain polyunsaturated fatty acids. The presence of large amounts of 20:5w3 appears to be characteristic of the lipids of the larger decapod Crustacea (see Addison, Ackman and Hingley, 1972) such as Jasus lalandii (De Koning and McMullen 1966), Cancer magister (Allen 1971) Callinectes sapidus (Whitney 1969) and Orconectes rusticus (Wolfe, Rao and Cornwell, 1968), rather than 22:6w3 which is characteristic of marine vertebrate lipids (Addison, Ackman and Hingley 1968, McMullen, Smith and Wright 1968).

The differences observed in the overall fatty acid distribution of the total phospholipid fraction from the abdominal muscle of crayfish acclimated to 4°C and to 25°C, were minor in extent. They consisted mainly of a smaller proportion of the saturated fatty acids in the former group, but slightly higher proportions

of the mono, di, tri, tetra and pentaenoic fatty acids.

The differences in the proportions of the polyunsaturated fatty acids were clearly not mediated by changes in the choline phosphoglyceride fraction since the proportions of these fatty acids in this fraction were very similar in the two lipid extracts. There was however a slightly lower proportion of the saturated fatty acids (16:0 and 18:0) in the choline phosphoglyceride and slightly higher proportions of the monoenoic and dienoic fatty acids in the extract from 4°C acclimated crayfish. In this respect the choline phosphoglycerides were relatively conservative.

The major difference in the polyenoic components of the total phospholipid fraction of the two extracts could be attributed to the acidic phospholipids. Both ethanolamine and serine/inositol phosphoglycerides showed clear increases in the proportion of the tetra and pentaenoic fatty acids in the extract from 4°C acclimated crayfish. This was caused largely by differences in the proportions of 20:4w6 and 20:5w3 in the serine/inositol phosphoglycerides and in 20:5w3 in the ethanolamine phosphoglyceride fatty acids.

An interesting but paradoxical feature of these results is the higher proportion of 22:6w3 in the phospholipids isolated from 25°C acclimated crayfish. This was caused almost exclusively by differences in the ethanolamine phosphoglyceride fraction. This phenomenon has not been observed in other studies, but the occurrence of significant quantities of this fatty acid in homoeotherm tissues (McMullen, Smith and Wright 1968, Klein 1970) suggests that docosahexaenoic acid may prove an exception to the general rule that the permeability of membranes and the unsaturated fatty acid content of membranes are positively related. This is in agreement with the aberrant behaviour of lecithins containing docosahexaenoic and palmitic acids, in respect of monolayer

packing characteristics and lipsome permeability (Demel, Geurts van Kessel and Van Deenen 1972).

In acclimation studies in general, there are a number of complicating factors, and the precise acclimation response observed may depend upon a variety of factors other than temperature. These include the acclimation procedure, the range of temperatures employed, the diet and frequency of feeding, seasonal influences and photoperiod. In the present study, these factors were controlled as far as possible by selecting each acclimation group from the same stock of animals. Each group was acclimated in identical ways. It is thought that the effect of physiological changes in the animals' biochemistry caused by moulting were minimised, since all animals were clearly in the intermoult condition throughout the whole procedure.

Under natural conditions, the two most important seasonal changes in the physical nature of the environment are temperature and photoperiod. In all the experiments discussed previously crayfish were acclimated at their respective temperatures with an 18 hr-artificial light photoperiod (i.e., 'summer' daylength) in an effort to control the effect of environmental factors upon their acclimation response. Subsequently, it was considered desirable to know whether photoperiod had any effect upon the acclimation response being studied.

It was evident from a comparison of the fatty acid composition of the total lipid fractions of 4°C and 25°C, 18-hr daylength acclimated crayfish and 4°C, 8-hr daylength acclimated crayfish, that the latter contained a higher unsaturated fatty acid content than the long-daylength acclimated animals. The response consisted mainly of a reduction in the proportion of saturated, mono, di and trienoic fatty acids in the former acclimation group. This was compensated by a marked increase in the proportion of tetra and pentaenoic fatty acids. The hexaenoic fraction comprised

identical proportions of the fatty acid composition of both extracts.

If, as suggested previously, choline phosphoglycerides are conservative with respect to modulation of membrane unsaturation, then most of these changes, especially those observed in the polyenoic fatty acid fractions would be expected to occur in the acidic phosphoglycerides. Since the ethanolamine and serine/inositol phosphoglycerides comprise only 25mole% and 10mole%, respectively, of the total phospholipids, then the effect on the fatty acid composition of these two phospholipids must be quite large to account for the observed differences in the total phospholipid fraction.

This interesting result leads to a further question. If the fatty acid composition of the muscle membrane does have some influence upon the heat death characteristics of the crayfish, and photoperiod and temperature induced greater changes in muscle fatty acid composition than is induced by temperature alone, do these two factors cause changes in the kinetics of heat death of the whole animal. Experiments were undertaken to investigate this problem, since photoperiod was not a factor that was considered or controlled by Bowler (1963a, b) or Gladwell (1973). The experimental details and results are presented in Figure 3-18.

In agreement with the results of Bowler (1963a) and Gladwell (1973), crayfish acclimated to 25°C (long daylength) were markedly more resistant to lethal high temperature stress than crayfish acclimated to 4°C (long daylength). However, although a few 4°C, short daylength animals died more rapidly at 32°C than 4°C, long daylength individuals, there was no marked difference between the overall heat death curves of the differently treated animals. The LD_{50} was 28 min. for 4°C, short daylength crayfish, and 28½ min. for 4°C, long daylength crayfish.

This latter observation has important consequences for the hypothesis that membrane unsaturation is an important factor in the

process of heat death in the crayfish and may be interpreted in several ways. Firstly, if the fatty acid analysis of short daylength acclimated crayfish presented earlier is correct and significantly different from the long daylength acclimated crayfish, then it indicates either that membrane unsaturation and thermal sensitity of the intact organism are not related or that the relationship is not proportional. The increase in the proportion of unsaturated fatty acids of membrane phospholipids, above that observed in the long-daylength acclimated crayfish does not produce any corresponding increase in the thermal sensitivity of the intact animal. Secondly, the fact that the analysis of short-daylength, 4°C acclimated crayfish was performed on the muscle from only three animals suggests that the results are not as representative of the acclimation group as in earlier analyses of the lipid extracts from ten crayfish. This possibility could only be tested by repeating the analysis to ensure that the biological variation in fatty acid composition between animals is not as great as the differences between acclimation groups. A third possibility is that the 4°C, long-daylength acclimated animals are undergoing heat death at 32°C so rapidly (LD_{50} = 28 minutes) that the rate cannot be increased significantly by acclimation to short daylength conditions. This factor could be studied by assessing the effect of short-daylength conditions upon 25°C acclimated crayfish, since it is evident that these animals are not undergoing heat death at the maximal rate.

The suggestion that photoperiod is involved in the control of membrane biochemistry has interesting implications. Firstly, as suggested in Chapter 1, it may impart to the animal concerned, the ability to "preadapt" its biochemistry and metabolism and anticipate the seasonal fluctuations in environmental conditions. Secondly, this phenomenon invokes the presence of a humoral factor in the control of membrane unsaturation, since it is considered

unlikely that crayfish muscle is directly responsive to changing daylength. It is considered more likely that photoreceptors in the compound eye receive the stimulus and the nervous system causes acclimatory responses via a neuroendocrine reflex.

Neuroendocrine mechanisms of this sort have been suggested by Hoar (1955) and Hoar and Robertson (1959) who found that goldfish maintained at constant temperature were more heat and cold resistant depending upon the photoperiod conditions experienced prior to the experiment. Johansen (1967) has suggested that this response in the goldfish is mediated by the hormones of the anterior pituitary. Sullivan and Fisher (1953) recorded a change of 5°C in the preferred temperature of the speckled trout Salvelinus sp. in anticipation of "the seasonal decline of water temperature with the approach of winter". Roberts (1964) has observed differences in the oxygen consumption/temperature curves of sunfish acclimated at the same temperature but different photoperiods. However, the sensitivity to photoperiod conditions in this animal and the goldfish (Hoar and Robertson 1959) is limited to specific phases of their breeding cycle. Seasonal cycles of endocrine activity in poikilothermic vertebrates is well documented and may be basic to the process of acclimation in these animals.

It is difficult to assess the impact of diet or of the differential incorporation of fat reserves into membrane phospholipids, upon the fatty acid composition of crayfish muscle phospholipids. In their studies on the American crayfish Orconectes rusticus, Wolfe, Rao and Cornwell (1968) have suggested that whilst triglycerides of the fat stores resemble the dietary lipid, the phospholipids mirror the biosynthetic pathways of the animal. In support of this statement, Addison, Ackman and Hingley (1968) and Roubal (1967) have concluded that the neutral lipids of cod flesh have relatively small proportions of polyunsaturates, but high proportions of saturated and monoenoic fatty acids. On the other hand, the fatty acid composition of the phospholipids

of rat erythrocytes (Walker and Kummerow, 1964) and brain and liver mitochondria (Wittering, Harvey, Century and Horwith, 1961) are known to be influenced by dietary fatty acids.

It is well known, however, that in animals the biosynthesis of polyunsaturated fatty acids can only proceed with dietary linoleic (18:2w6) and linolenic (18:3w3) fatty acids due to certain enzymic deficiencies (Gurr and James, 1971). The availability of these fatty acids in the diet or in depot fats might affect the acclimation characteristics of an animal. The fact that warm-acclimated crayfish / appear to have a higher metabolic rate (Bowler 1963b) / may have some relevance upon the rate of dietary incorporation of fatty acid into phospholipids. It is possible to construct biosynthetic pathways for the production of all polyenoic fatty acids identified in crayfish muscle (Figure 3-17). The intermediate fatty acids for these pathways (with the exception of 22:3w9) have been identified during the initial analysis of choline phosphoglyceride fatty acids.

It is tentatively concluded, therefore, that acclimation to lower temperatures at constant daylength, causes an increase in the overall unsaturation of the muscle phospholipids. This is mediated mainly by changes in the acidic phospholipids. A word of caution, however, is urged since in order to be absolutely certain that this effect is real, the analysis should be repeated to ensure that the biological variation in the fatty acid composition between crayfish of the same acclimation group is not as great as the differences between the different acclimation groups.

This conclusion is in broad agreement with the results of a number of other studies (see Table 6-15). Perhaps the most detailed analysis of the effect of thermal acclimation upon the fatty acid composition of poikilotherms, is that of Kemp and Smith (1970) on the goldfish. Whilst the fatty acid composition of whole-fish lipids was not markedly affected by alteration of

acclimation temperature, there were large differences in the fatty acid composition of lipids extracted from the homogenates of intestinal mucosa from differently acclimated goldfish. For example, raising the acclimation temperature by 20°C effectively halved the proportions of 20:1, 20:4 and 22:6 fatty acids, whilst the proportions of 18:0 and 20:3 were doubled. In contrast to the conclusions of the present study, Kemp and Smith noted that the fatty acids of serine and inositol phosphoglycerides were less susceptible to acclimation-dependent changes than the choline and ethanolamine phosphoglycerides. It is suggested that because of the different specialisation of their component membranes, different tissues might adapt to temperature changes in different ways (cf. Roots, 1968).

In a similar analysis of the effects of thermal acclimation upon the fatty acid composition of the total lipid extract from goldfish brain, Johnson and Roots (1964) reported an increase in the unsaturation of the fatty acids when the acclimation temperature was decreased. This effect was mediated mainly by changes in the relative amounts of stearic (18:0), arachidonic (20:4) and docosapenta (and hexa) enoic acids (22:5, 22:6). Less pronounced alterations occurred in the proportions of palmitoleic (16:1), linoleic (18:2) and linolenic (18:3) fatty acids. However, these results are in contrast to a more recent communication, where Roots (1968) fractionated the brain phospholipids and analysed the fatty acid composition of choline and ethanolamine phosphoglycerides separately. Although the overall fatty acid unsaturation increased with decreased acclimation temperature, this was caused by changes in the C16 and C18 fatty acids. There was no consistent or significant change in the proportion of polyunsaturated fatty acids of either phospholipids. Kemp and Smith (1970) have suggested that seasonal factors and selective autoxidation of the polyunsaturates may account for some of these inconsistencies.

The plasmalogen content and composition of goldfish brain phospholipids were analysed by Roots and Johnson (1968). The results indicated a striking decrease in the proportion of plasmalogen phospholipids in comparison to the diacyl variety, in response to a decrease in acclimation temperature. Furthermore the compositional analysis of the dimethyl acetal derivatives of phosphatidal ethanolamine showed that at lower acclimation temperatures there was a greater degree of unsaturation. However, lack of detailed knowledge concerning the membrane characteristics of plasmalogens precludes a valid discussion of the relevance of these findings to thermal acclimation.

Baranska and Wlodawer (1966) have examined the same phenomenon in frog liver. Although they demonstrated marked changes in the fatty acid composition at different acclimation temperatures, their analysis fails to identify several important polyunsaturated fatty acids that have been identified in a closely-related species (McMullin, Smith and Wright, 1968) and their conclusions should be viewed with some caution.

Knipprath and Meads (1966) analysed the total lipids fraction of the Mosquitofish (Gambusia sp.) and the Guppy (Lebistes sp.) that had been acclimated at 14-15°C or 26-27°C for 14 days. In the case of mosquitofish they claim "distinct trends to higher unsaturation of the fatty acids at lower temperatures". Their data reveals only small differences in the unsaturation of the fatty acids of the two acclimation groups. In addition, interpretation of their results is complicated by the absence of repeated analyses of the lipid sample to determine whether the observed differences are due to biological and not experimental variation.

The adaptive properties of these described responses have usually been given in terms of the maintenance of the appropriate degree of 'fluidity' of the liquid-crystalline membrane, that is necessary for its correct and efficient function at the

environmental temperature. However, this compensatory effect has not been demonstrated experimentally for any poikilothermic animal, although Haest, de Gier and Van Deenen (1969) have shown an increase in the unsaturation of membrane lipids of E. coli in response to growth at lower temperatures. This correlates well with a more expanded monolayer and increased liposome permeability of these phospholipids. Cullen, Phillips and Shipley (1971) have made similar observations with Pseudomonas fluorescens. These facts point to a control mechanism of the physical state of the lipids that is sensitive to the environmental temperature and/or membrane fluidity.

The precise physico-chemical significance of the reported increase in the proportion of polyenoic fatty acids in membrane phospholipids is difficult to assess in view of the lack of information concerning the membrane properties of highly polyunsaturated phospholipids. Confirmation of the adaptive nature of this response is necessary, and could most easily be achieved with 'liposome' model membrane systems prepared from the purified phospholipids.

In addition to providing the major permeability barrier between a cell and its environment, cellular membranes also provide an appropriate framework for certain membrane-bound enzyme systems. It has been proposed that certain enzymes, particularly those involved in ion transport across membranes, involve sequential changes in the folding and unfolding of the protein moeity (Opit and Charnock, 1965; Duncan 1967). An increase in the proportion of unsaturated fatty acids of membrane phospholipids at lower acclimation temperatures, may be expected to cause less restraint upon the conformational flexibility of the enzyme and permit more rapid membrane transport, thereby compensating for the effects of reduced kinetic energy. The effect of membrane composition upon membrane-bound enzyme activity has been

demonstrated most convincingly in micro-organisms. For example, Wilson and Fox (1971) have shown that fatty acid auxotrophs of E. coli grown on oleate-enriched medium, exhibited a distinct break in their Arrhenius plots at 13-14°C for the glucose and galactose transport systems, whereas cells grown on linoleate-enriched medium were characterised by a break at 7-8°C. These transitional temperatures correspond approximately with the phase transitions of the respective fatty acids.

Smith and Ellory (1971) have found that the decrease in (Na^+-K^+)-ATPase activity that occurs during warm-acclimation is not caused by a decreased number of pump sites as measured by ouabain binding. Similarly, an increased respiratory activity of gill mitochondria of cold-acclimated goldfish was not accompanied by changes in the cytochrome c or cytochrome oxidase concentration (Caldwell, 1969). This evidence strongly implies that the activity rather than the overall quantity of these enzyme is being regulated during thermal acclimation.

This hypothesis is supported by studies of succinate dehydrogenase in the muscle of the goldfish (Hazel, 1972, b). Reactivation of the delipidated enzyme preparation with phospholipids extracted from cold-acclimated goldfish muscle resulted in a greater SDH activity than when the preparation was reactivate with phospholipids isolated from warm-acclimated goldfish. In addition, many membrane-bound enzymes exhibit a partial or total requirement for unsaturated fatty acids for their activity (Rothfield and Finkelstein, 1968; Cunningham and Hager, 1971; Coleman, 1973).

Table 3-1: The structure and commercial source of the authentic fatty acids methyl esters used for the calibration of the chromatographic system, and for the identification of unknown methyl esters

Legend:

*	%wt composition (manufacturers' data)
**	Compositional data not available.
✓	Present

Table 3-1

Source		SIGMA									Applied Science	SUPELCO	
Fatty Acid Methyl Ester	Trivial name	1 *	189-1 *	189-2 *	189-3 *	189-4 *	189-5 *	189-6 *	D4257 *	E0875 *	L207 **	PUFA 2 **	RM-1 *
8:0	Caprylate				20								
10:0	Caprate				20								
12:0	Laurate				20								
13:0	Tridecanoate							20					
14:0	Myristate				20								
15:0	Pentadecanoate							20					
16:0	Palmitate	99+	20	20	20		25						60
16:1	Palmitoleic					25							
17:0	Heptadecanoate							20					
18:0	Stearate		20	20			25				✓		30
18:1	Oleate		20	20		25					✓		350
18:2	Linoleate		20									✓	500
18:3	Linolenate		20	20								✓	30
19:0	Nonadecoate							20					
20:0	Arachidate			20			25				✓		30
20:1	Eicosanoate					25							
20:4	Eicosatetraenoate									99+		✓	
20:5	Eicosapentaenoate											✓	
21:0	Heneicosanoate							20					
22.0	Behenate						25				✓		
22:1	Erucate					25					✓		
22:4	Docosatetraenoate											✓	
22:5	Docosapentaenoate											✓	
22:6	Docosahexenoate								99+				
24:0	Ligneocrate										✓		
24:1	Nervonate										✓		

Table 3-2: The Relative Retention Times of Fatty Acid Methyl Esters determined using Authentic Standards and from the Literature

Data from Ackman (1963a, b) and Burchfield and Storrs (1962)

	Fatty Acid Structure	RRt values		
		Ackman(1963)	Burchfield and Storrs	Present Data
Monoene	16:1w9	1.14	-	
	18:1w9	2.04	2.04	
	20:1w9	3.68	3.67	
	22:1w9	6.70	-	
	24:1w9			11.94
	16:1w7	1.151	1.15	1.16
Diene	16:2w4	1.472	1.43	
	18:2w4	2.64	-	
	18:2w5	2.49	-	
	16:2w6	1.355	-	
	18:2w6	2.44	2.44	
	20:2w6	4.46	4.46	
	22:2w6	7.96	-	
	16:2w7	1.346	1.43	
	18:2w7	2.42	-	
	22:2w7	7.63	-	
	18:2w9	2.35	2.60	
	20:2w9	4.22	4.22	
Triene	16:3w3	1.731	1.64	
	18:3w3	3.13	3.13	3.12
	16:3w4	1.642	1.64	
	16:3w6	1.565	-	
	18:3w6	2.80		
	20:3w6	5.02	5.02	5.04
Tetraene	16:4w1	2.02	-	
	16:4w3	1.965	-	
	18:4w3	5.58	3.58	
	20:4w3	6.39	-	
	20:4w6	5.52	5.53	5.57
	22:4w6	10.00	-	10.07
Pentaene	20:5w3	7.00	7.00	6.95
	22:5w3	12.75	12.73	12.75
	22:5w4	12.00	12.00	
	22:5w6	11.06	11.07	
Hexaene	22:6w3	14.09	14.09	

Table 3-3: Summary of the Relative Retention Times (16:0) of fatty acid methyl esters, determined by chromatography, authentic standard or by calculation using the 'separation factors' or 'semilog plot' procedures

Method: See 'Materials and Methods'.

Legend:
- * From Ackman (1963a).
- □ Burchfield and Storrs (1962)
- + Predicted from semilog plot.
- o Calculated by separation factor procedures.

Table 3-3

No. of Carbon Atoms	End Carbon chain	NUMBER OF OLEFINIC BONDS						
		0	1	2	3	4	5	6
16	– W3 W4 W6 W7 W9	1.00	 1.160 *□ 1.144	 1.472 * 1.355 * 1.346	1.731 * 1.642 *□ 1.565 *	1.965 * 1.67 +		
17	– W4 W9	1.37 *□+	1.51 +	 1.73 + 1.98 +				
18	– W3 W4 W6 W7 W9	1.82 *□+	 2.04 *□+	 2.64 * 2.44 * 2.42 * 2.35	3.13 *□ 2.80 * 2.69 °	3.58 *□ 3.28 ° 3.02 + 2.91 °		
19	– W9	2.46 +□	2.72 +	3.14 +				
20	– W3 W6 W9	3.31 +□	 3.68 *	 4.99 ° 4.46 *□ 4.22	5.67 * 5.02 *□ 4.78 °	6.39 * 5.52 *□ 5.26 °	7.04 *□ 6.12 ° 5.83 °	
21	– W9	4.47 +□	4.91 +	5.68 +				
22	– W3 W4 W6 W9	5.95 +□	 6.70 *	 8.60 + 7.96 * 7.63 +	10.35 ° 9.15 * 8.56 °	11.70 * 10.01 * 9.70 *	12.75 *□ 12.00 *□ 11.06 * 10.51 °	14.09 *□ 12.21 ° 11.65 °
24	– W9	11.16 +□	11.94 +□					

Table 3-4: Summary of the fatty acid methyl esters of choline phosphoglycerides identified by gas-liquid chromatography after 'argentation' TLC. The RRt of the chromatographic peak and for the predicted methyl ester are shown for comparison

Method: See 'Materials and Methods'

Legend: * Minor or trace components

** Predicted RRt (Table 3-3)

Table 3-4

Number of unsaturation bonds	Fatty acid species	Predicted RRt **	FRACTION NUMBERS				
			I	II	III	IV	V
0	16:0	1.00	1.00				
	17:0	1.37	1.37*				
	18:0	1.83	1.83				
	19:0	2.46					
	20:0	3.31					
1	16:1w9	1.14	1.14	1.14*			
	17:1w9 (iso 16:0)	1.51	1.51*				
	18:1w9	2.04	2.05	2.05			
	19:1w9	2.72	2.77*				
	20:1w9	3.68	3.73		3.67		
2	16:2w6	1.36		1.42*			
	18:2w6	2.44		2.46			
	20:2w3	4.92		4.99*			
	20:2w6	4.48		4.46			
	20:2w9	4.22		4.12*		4.23*	
	22:2w9	7.63		7.45*			
3	18:3w3	3.13			3.13		
	18:3w6	2.80			2.78*		
	20:3w3	5.67			5.71		
	20:3w6	5.02			5.01*		
	20:3w9	4.78			4.82*		
	22:3w6	9.15			9.00*		
4	16:4w6	1.67				1.67*	
	18:4w3	3.58					3.45
	18:4w6	3.02				3.07*	
	20:4w6	5.52				5.51	
	20:4w3	6.39				6.40*	
	22:4w6	10.01				10.02*	
5	20:5w6	6.40					6.12*
	20:5w3	7.03					7.04
	22:4w9	9.70					9.85
	22:5w6	11.06					11.20
	22:5w3	12.75					12.70
6	22:6w3	14.09					14.19

Table 3-5: The fatty acid composition of the total phospholipid fraction of the lipid extracts from crayfish acclimated to 4°C and 25°C, under 18 hour-light photoperiod conditions

Methods: Each lipid extract was analysed several times and the fatty acid composition determined as described in the text.

* The values reported are the % total weight (= peak area) and are the average ± S.E.M. of the replicate chromatographic analyses.

The RRt. determined for authentic fatty acid methyl esters and for unavailable methyl esters by the 'semilog' and 'separation factor' procedures (Table 3-3), are included for comparison with the RRt. of the components of the samples.

** Values taken from Table 3-3.

* Trace component (0.1%)

Table 3-5

Observed RRt	Identified Fatty Acid	Predicted RRt **	4°C Acclimated Crayfish (n=5)	25°C Acclimated Crayfish (n=5)
			18 hour Daylength	18 hour Daylength
1.00	16:0	1.00	10.07 ± 0.16	11.59 ± 9.18
1.16	16:1w9	1.14	3.94 ± 0.05	3.67 ± 0.10
1.35	17:0	1.37	0.57 ± 0.04	0.81 ± 0.01
1.55	17:1w9 (iso 16:0)	1.51	1.47 ± 0.37	1.04 ± 0.01
1.63	16:4w6	1.67		0.87 ± 0.08
1.72	17:2w4	1.73	0.35 ± 0.08	
1.83	18:0	1.82	4.45 ± 0.04	6.58 ± 0.11
2.06	18:1w9	2.04	18.08 ± 0.15	18.45 ± 0.15
2.34	18:2w9	2.34		
2.46	18:2w6	2.44	4.67 ± 0.05	3.01 ± 0.02
2.80	18:3w6	2.80	0.39 ± 0.01	0.37 ± 0.01
3.09	18:3w3	3.13	1.29 ± 0.01	1.16 ± 0.01
3.43	18:4w3	3.58	0.14 ± 0.01	0.25 ± 0.02
3.75	20:1w9	3.68	1.68 ± 0.03	1.33 ± 0.01
4.23	20:2w9	4.22		tr
4.50	20:2w6	4.46	1.44 ± 0.02	1.58 ± 0.01
5.08	20:3w6	5.02	0.36 ± 0.09	0.27 ± 0.04
5.54	20:4w6, 20:3w3	5.52	10.54 ± 0.19	9.70 ± 0.12
6.17	20:5w6	6.12	0.31 ± 0.03	0.37 ± 0.06
7.04	20:5w3	7.04	31.69 ± 0.17	30.16 ± 0.67
7.68	22:2w9	7.63	0.11 ± 0.00	0.10 ± 0.01
8.24	22:2w6	7.96	0.08 ± 0.00	0.10 ± 0.02
9.31	22:3w6	9.15	0.25 ± 0.04	0.28 ± 0.05
9.83	22:4w9	9.70	0.53 ± 0.00	0.68 ± 0.02
10.48	22:5w9	10.51	0.10 ± 0.02	0.17 ± 0.01
11.27	22:5w6	11.06	0.49 ± 0.03	0.42 ± 0.01
12.89	22:5w3	12.75	1.40 ± 0.07	1.20 ± 0.05
14.18	22:6w3	14.19	4.33 ± 0.04	5.48 ± 0.04
Others	-		0.75 -	0.34 -

Table 3-6: The carbon chain length (a) and fatty acid unsaturation (b) class distribution of total phosphoglyceride fraction from the muscle of 4°C and 25°C acclimated crayfish maintained under 18 hour-light photoperiod conditions

* Values reported are the % total weight (= peak area) for each class and are the average of ± S.E.M. of replicate analyses (see Table 3-5).

Table 3-6a

Carbon Chain Length	16	17	18	20	22	Others
4°C Long Daylength (n=5)	13.90 ± 0.22* (13.44-14.51)	2.04 ± 0.38 (0.72-3.04)	29.74 ± 0.18 (29.82-30.42)	46.00 ± 0.40 (44.68-46.83)	7.04 ± 0.12 (6.79-7.40)	1.20 ± 0.28 (0.5-2.11)
25°C Long Daylength (n=5)	15.27 ± 0.28 (14.46-15.99)	1.85 ± 0.02 (1.77-1.93)	29.82 ± 0.11 (29.52-30.09)	43.41 ± 0.91 (42.72-43.81)	8.51 ± 0.17 (8.15-8.96)	1.09 ± 0.18 (0.68-1.59)
t	3.847	0.499	0.381	5.850	7.070	0.831
P	0.001-0.01	0.6-0.7	0.7-0.8	<0.001	<0.001	0.4-0.5

Table 3-6b

Number of Olefinic Bonds	0	1	2	3	4	5	6	Others
4°C Long Daylength (n=5)	14.99 ± 0.23* (14.61-15.61)	25.37 ± 0.33 (24.79-26.60)	6.29 ± 0.07 (6.14-6.54)	2.05 ± 0.05 (1.83-2.27)	11.21 ± 0.19 (10.53-11.62)	33.99 ± 0.24 (33.19-34.39)	4.33 ± 0.04 (4.20-4.45)	1.28 ± 0.31 (0.51-2.13)
25°C Long Daylength (n=5)	18.99 ± 0.12 (18.59-19.35)	24.50 ± 0.21 (23.96-25.08)	4.80 ± 0.05 (4.65-4.92)	1.80 ± 0.05 (1.66-1.95)	10.90 ± 0.10 (10.55-11.13)	32.34 ± 0.14 (32.00-32.55)	5.48 ± 0.04 (5.38-5.58)	1.09 ± 0.18 (0.68-1.40)
t	15.420	3.503	17.326	2.651	1.444	5.930	20.350	0.530
P	<0.001	0.01-0.001	<0.001	0.02-0.05	0.1-0.2	<0.001	<0.001	0.6-0.7

Table 3-7: The fatty acid composition of the choline phospholipid fraction of the lipid extracts from crayfish acclimated to 4°C and 25°C, under 18 hour-light photoperiod conditions

Methods: Each lipid extract was analysed several times and the fatty acid composition determined as described in the text. The values reported are the % total weight (= peak area) and are the average ± S.E.M. of the replicate analyses.

The RRt. determined for authentic fatty acid methyl esters and for unavailable methyl esters by the 'semilog' and 'separation factor' procedures (Table 3-3), are included for comparison with the RRt. of the components of the samples.

** Values taken from Table 3-3

tr Trace component

Table 3-7

Observed RRt	Identified Fatty Acid	Predicted RRt **	4°C Acclimated Crayfish	25°C Acclimated Crayfish
			18 hour Daylength	18 hour Daylength
1.00	16:0	1.00	16.70 ± 0.40	18.67 ± 0.22
1.16	16:1w9	1.14	6.74 ± 0.15	5.97 ± 0.08
1.35	17:0,	1.37	0.60 ± 0.05	0.82 ± 0.04
1.55	17:1w9 (iso 16:0)	1.51	1.30 ± 0.02	7.13 ± 0.02
1.63	16:4w6	1.67	-	-
1.72	17:2w4	1.73	-	-
1.83	18:0	1.82	2.29 ± 0.08	4.18 ± 0.04
2.06	18:1w9	2.04	25.85 ± 0.24	25.89 ± 0.66
2.34	18:2w9	2.34	-	-
2.46	18:2w6	2.44	6.78 ± 0.05	3.74 ± 0.09
2.80	18:3w6	2.80	0.46 ± 0.01	0.47 ± 0.00
3.09	18:3w3	3.13	1.85 ± 0.03	1.37 ± 0.03
3.43	18:4w3	3.58	0.12 ± 0.02	0.12 ± 0.00
3.75	20:1w9	3.68	1.77 ± 0.03	1.45 ± 0.03
4.23	20:2w9	4.22	0.09 ± 0.03	0.08 ± 0.02
4.50	20:2w6	4.46	1.44 ± 0.05	1.70 ± 0.05
5.08	20:3w6	5.02	0.30 ± 0.04	0.33 ± 0.05
5.54	20:4w6, 20:3w3	5.52	7.20 ± 0.04	6.81 ± 0.06
6.17	20:5w6	6.12	tr	0.18 ± 0.04
7.04	20:5w3	7.04	22.55 ± 0.48	22.40 ± 0.19
7.68	22:2w9	7.63	0.18 ± 0.02	0.08 ± 0.02
8.24	22:2w6	7.96	0.01 ± 0.00	0.15 ± 0.06
9.31	22:3w6	9.15	0.11 ± 0.02	tr
9.83	22:4w9	9.70	0.15 ± 0.00	0.36 ± 0.12
10.48	22:5w9	10.51	0.06 ± 0.00	0.10 ± 0.03
11.27	22:5w6	11.06	0.23 ± 0.02	0.13 ± 0.01
12.89	22:5w3	12.75	0.64 ± 0.08	0.84 ± 0.15
14.18	22:6w3	14.19	2.40 ± 0.08	2.25 ± 0.45
Others	-	-	0.24 -	0.94 -

Table 3-8: The carbon chain length (a) and fatty acid unsaturation (b) class distribution of the choline phosphoglyceride fraction from the muscle of 4°C and 25°C acclimated crayfish maintained under 18 hour-light photoperiod conditions

* Values reported are the % total weight (= peak area) for each class and are the average ± S.E.M. of replicate analyses (see Table 3-7).

Table 3-8a

Carbon Chain Length	16	17	18	20	22	Others
4°C Long Daylength (n=4)	23.44 ± 0.47* (22.57-24.45)	1.89 ± 0.05 (1.81-2.01)	37.35 ± 0.23 (36.73-37.78)	33.37 ± 0.53 (32.50-34.86)	3.69 ± 0.09 (3.48-3.88)	0.30 ± 0.06 (0.20-0.44)
25°C Long Daylength (n=4)	24.61 ± 0.23 (24.00-25.06)	1.94 ± 0.07 (1.82-2.10)	35.77 ± 0.72 (33.92-37.30)	32.85 ± 0.20 (32.38-33.35)	3.89 ± 0.71 (2.80-5.92)	0.72 ± 0.22 (0.10-1.11)
t	2.240	0.581	2.090	0.940	0.279	1.842
P	0.05-0.1	0.5-0.6	0.05-0.1	0.3-0.4	0.7-0.8	0.1-0.2

Table 3-8b

Number of Olefinic Bonds	0	1	2	3	4	5	6	Others
4°C Long Daylength (n=4)	19.58 ± 0.50* (18.38-20.72)	35.66 ± 0.16 (35.18-35.37)	8.50 ± 0.09 (8.25-8.62)	2.61 ± 0.00 (2.59-2.63)	7.47 ± 0.04 (7.36-7.58)	23.47 ± 0.49 (22.96-24.79)	2.40 ± 0.08 (2.26-2.62)	0.30 ± 0.05 (0.20-0.44)
25°C Long Daylength (n=4)	23.67 ± 0.49 (23.32-24.22)	34.41 ± 0.70 (32.78-35.89)	5.74 ± 0.10 (5.54-6.03)	2.17 ± 0.03 (2.10-2.25)	7.29 ± 0.15 (6.99-7.68)	23.55 ± 0.18 (23.14-24.03)	2.45 ± 0.45 (1.74-3.58)	0.72 ± 0.02 (0.10-0.11)
t	5.843	1.741	16.019	14.667	1.160	0.153	0.109	1.842
P	0.001-0.01	0.1-0.2	<0.001	<0.001	0.2-0.3	0.8-0.9	0.9-1.0	0.1-0.2

Table 3-9: The fatty acid composition of the ethanolamine phosphoglyceride fraction of the lipid extracts from crayfish acclimated to 4°C and 25°C, under 18 hour-light photoperiod conditions

Method: Each lipid extract was analysed several times, and the fatty acid composition determined as described in the text.

*The values reported are the % total weight (= peak area) and are the average ± S.E.M. of the replicate analyses.

The RRt determined for authentic fatty acid methyl esters and for unavailable methyl esters by the 'semilog' and 'separation factor' procedures (Table 3-3) are included for comparison with the RRt. of the components of the samples.

** Values taken from Table 3-3.

tr Trace component

Table 3-9

Observed RRt	Identified Fatty Acid	Predicted RRt **	4°C Acclimated Crayfish	25°C Acclimated Crayfish
			18 hour Daylength	18 hour Daylength
1.00	16:0	1.00	3.32 ± 0.21	6.41 ± 0.40
1.16	16:1w9	1.14	2.38 ± 0.08	1.96 ± 0.14
1.35	17:0	1.37	0.15 ± 0.01	0.34 ± 0.05
1.55	17:1w9 (iso 16:0)	1.51	5.44 ± 0.80	3.31 ± 0.55
1.63	16:4w6	1.67		
1.72	17:2w4	1.73	2.12 ± 0.15	1.74 ± 0.17
1.83	18:0	1.82	1.32 ± 0.12	2.31 ± 0.19
2.06	18:1w9	2.04	15.15 ± 0.17	14.80 ± 0.40
2.34	18:2w9	2.34		
2.46	18:2w6	2.44	3.68 ± 0.08	2.90 ± 0.24
2.80	18:3w6	2.80	0.36 ± 0.02	0.32 ± 0.05
3.09	18:3w3	3.13	0.88 ± 0.00	0.81 ± 0.03
3.43	18:4w3	3.58	0.04 ± 0.00	0.07 ± 0.00
3.75	20:1w9	3.68	1.38 ± 0.04	1.19 ± 0.07
4.23	20:2w9	4.22	0.08 ± 0.00	0.08 ± 0.01
4.50	20:2w6	4.46	0.96 ± 0.00	1.28 ± 0.22
5.08	20:3w6	5.02	0.26 ± 0.04	0.35 ± 0.05
5.54	20:4w6, 20:3w3	5.52	11.29 ± 0.17	10.50 ± 0.12
6.17	20:4w6 20:5w6	6.12	0.49 ± 0.07	0.41 ± 0.12
7.04	20:5w3	7.04	39.20 ± 0.73	35.82 ± 0.48
7.68	22:2w9	7.63	0.15 ± 0.01	0.15 ± 0.02
8.24	22:2w6	7.96	0.14 ± 0.02	0.34 ± 0.08
9.31	22:3w6	9.15	0.12 ± 0.02	0.20 ± 0.07
9.83	22:4w9	9.70	0.78 ± 0.17	0.51 ± 0.15
10.48	22:5w9	10.51		0.21 ± 0.04
11.27	22:5w6	11.06	0.76 ± 0.01	0.79 ± 0.04
12.89	22:5w3	12.75	1.79 ± 0.04	1.80 ± 0.08
14.18	22:6w3	14.19	7.37 ± 0.16	10.53 ± 0.36
Others	-	-	tr	1.09 -

Table 3-10: The carbon chain length (a) and the fatty acid unsaturation (b) class distribution of the ethanolamine phosphoglyceride fraction from the muscle of 4°C and 25°C acclimated crayfish, maintained under 18 hour-light photoperiod conditions

* Values reported are the % total weight (= peak area) for each class and are the average ± S.E.M. of replicate analyses (see Table 3-9).

Table 3-10a

Carbon Chain Length	16	17	18	20	22	Others
4°C Long Daylength (n=5)	5.68 ± 0.27 (5.10-6.35)	5.68 ± 0.81 (2.76-7.14)	21.43 ± 0.33 (20.47-22.44)	53.55 ± 0.80 (51.74-55.99)	10.99 ± 0.22 (10.21-11.55)	2.43 ± 0.19 (1.74-2.83)
25°C Long Daylength (n=4)	8.37 ± 0.50 (7.46-9.35)	3.64 ± 0.58 (2.39-5.06)	21.21 ± 0.72 (19.50-22.90)	49.63 ± 0.63 (48.35-50.85)	14.29 ± 0.60 (13.88-15.79)	2.66 ± 0.22 (2.22-3.08)
t	4.734	2.048	0.278	3.850	5.164	0.791
P	0.001-0.01	0.05-0.1	0.7-0.8	0.001-0.01	0.001-0.01	0.4-0.5

Table 3-10b

Number of Olefinic Bonds	0	1	2	3	4	5	6	Others
4°C Long Daylength (n=5)	4.89 ± 0.33 (4.26-5.92)	24.35 ± 0.71 (21.82-25.67)	4.99 ± 0.10 (4.74-5.24)	1.50 ± 0.07 (1.34-1.67)	12.12 ± 0.16 (11.75-12.59)	42.14 ± 0.65 (40.60-44.14)	7.37 ± 0.16 (7.04-7.89)	2.43 ± 0.19 (1.74-2.83)
25°C Long Daylength (n=4)	9.15 ± 0.61 (7.78-10.45)	21.26 ± 0.50 (20.20-22.20)	4.65 ± 0.16 (4.40-5.11)	1.47 ± 0.11 (1.28-1.75)	11.08 ± 0.25 (10.50-11.74)	39.02 ± 0.62 (37.69-40.31)	10.53 ± 0.36 (9.73-11.38)	2.66 ± 0.22 (2.22-3.68)
t	6.114	3.559	1.803	0.230	3.504	3.474	8.02	0.791
P	<0.001	0.01-0.001	0.1-0.2	0.8-0.9	0.001-0.1	0.01-0.02	<0.001	0.4-0.5

Table 3-11: The fatty acid composition of the serine/ inositol phosphoglyceride fraction of the lipid extracts from crayfish acclimated to 4°C and 25°C, under 18 hour-light photoperiod conditions

Methods: Each lipid extract was analysed several times and the fatty acid composition determined as described in the text.

*The values reported are the % total weight (= peak area) and are the average ± S.E.M. of the replicate analyses.

The RRt determined for authentic fatty acid methyl esters and for unavailable methyl esters by the 'semilog' and 'separation factor' procedures (Table 3-3), are included for comparison with the RRt. of the components of the samples.

** Values taken from Table 3-3.

tr Trace component

Table 3-11

RRt	Fatty Acid	Predicted RRt **	4°C Acclimated Crayfish	25°C Acclimated Crayfish
			18 hour Daylength	18 hour Daylength
1.00	16:0	1.00	4.33 ± 0.38	4.13 ± 0.48
1.16	16:1w9	1.14	0.62 ± 0.17	1.07 ± 0.55
1.35	17:0	1.37	1.79 ± 0.15	2.16 ± 0.06
1.55	17:1w9 (iso 16:0)	1.51	-	-
1.63	16:4w6	1.67	0.31 ± 0.05	0.29 ± 0.03
1.72	17:2w4	1.73	-	-
1.83	18:0	1.82	18.72 ± 0.79	25.62 ± 0.94
2.06	18:1w9	2.04	6.97 ± 0.19	5.23 ± 0.24
2.34	18:2w9	2.34	0.17 ± 0.01	0.33 ± 0.17
2.46	18:2w6	2.44	0.59 ± 0.03	0.43 ± 0.14
2.80	18:3w6	2.80	0.53 ± 0.09	0.48 ± 0.02
3.09	18:3w3	3.13	0.10 ± 0.03	0.13 ± 0.03
3.43	18:4w3	3.58	0.45 ± 0.06	0.60 ± 0.11
3.75	20:1w9	3.68	1.75 ± 0.10	1.34 ± 0.16
4.23	20:2w9	4.22	0.09 ± 0.03	0.35 ± 0.07
4.50	20:2w6	4.46	1.45 ± 0.12	0.97 ± 0.06
5.08	20:3w6	5.02	0.83 ± 0.11	0.85 ± 0.19
5.54	20:4w6, 20:3w3	5.52	19.21 ± 0.25	14.71 ± 0.72
6.17	20:5w6	6.12	0.20 ± 0.09	0.42 ± 0.14
7.04	20:5w3	7.04	37.21 ± 0.78	33.11 ± 0.54
7.68	22:2w9	7.63	0.26 ± 0.09	0.23 ± 0.04
8.24	22:2w6	7.96	0.11 ± 0.05	0.47 ± 0.23
9.31	22:3w6	9.15	0.34 ± 0.03	0.38 ± 0.13
9.83	22:4w9	9.70	-	-
10.48	22:5w9	10.51	0.10 ± 0.00	tr
11.27	22:5w6	11.06	0.50 ± 0.10	0.69 ± 0.24
12.89	22:5w3	12.76	0.97 ± 0.13	1.28 ± 0.38
14.18	22:6w3	14.19	1.37 ± 0.19	1.93 ± 0.13
Others	-	-	tr	1.67 -

Table 3-12: The carbon chain length (a) and the fatty acid unsaturation (b) class distribution of the serine/inositol phosphoglyceride fraction from the muscle of $4^{o}C$ and $25^{o}C$ acclimated crayfish

* Values reported are the % total weight (= peak area) for each class and are the average $\pm$ S.E.M. of replicate analyses (see Table 3-11).

Table 3-12a

Carbon Chain Length	16	17	18	20	22	Others
4°C Long Daylength (n=4)	4.95 ± 0.51* (4.14-6.44)	2.10 ± 0.18 (1.58-2.37)	27.36 ± 0.78 (25.46-28.43)	59.95 ± 0.69 (58.07-61.17)	3.30 ± 0.49 (2.23-4.54)	1.10 ± 0.30 (0.47-1.89)
25°C Long Daylength (n=3)	5.20 ± 0.91 (3.62-6.76)	2.16 ± 0.06 (2.03-2.22)	32.78 ± 0.80 (31.35-34.10)	51.71 ± 0.87 (50.57-53.43)	4.59 ± 0.73 (3.70-6.03)	3.27 ± 0.09 (3.18-3.44)
t	0.240	0.316	4.851	7.421	1.467	6.928
P	0.8-0.9	0.7-0.8	0.001-0.01	<0.001	0.2-0.3	<0.001

Table 3-12b

Number of Olefinic Bonds	0	1	2	3	4	5	6	Others
4°C Long Daylength (n=4)	24.84 ± 1.18* (21.78–26.76)	9.65 ± 0.28 (9.16–10.41)	2.48 ± 0.05 (2.38–2.61)	1.46 ± 0.16 (1.10–1.88)	19.76 ± 0.26 (19.00–20.21)	38.89 ± 0.88 (36.64–40.76)	1.37 ± 0.19 (0.91–1.77)	1.10 ± 0.30 (0.89–1.89)
25°C Long Daylength (n=3)	31.91 ± 0.59 (30.79–32.77)	7.64 ± 0.73 (6.61–9.06)	2.75 ± 0.34 (2.32–3.41)	1.46 ± 0.19 (1.12–1.77)	15.31 ± 0.64 (14.53–16.58)	35.77 ± 0.42 (34.77–36.22)	1.93 ± 0.13 (1.79–2.19)	3.27 ± 0.09 (3.18–3.44)
t	5.359	2.571	0.786	0.000	6.44	3.487	2.433	6.928
P	0.001–0.01	0.05	0.4–0.5	1.000	0.001–0.01	0.01–0.02	0.05–0.10	<0.001

Table 3-13: The fatty acid composition of the total phospholipid fraction of the lipid extract from the muscle of crayfish acclimated to $4^{o}C$, under 8 hour-light photoperiod conditions

Methods: Each lipid extract was analysed several times and the fatty acid composition determined as described in the text.

*The values reported are % total weight (= peak area) and are the average $\pm$ S.E.M. of the replicate analyses.

The RRt. determined for authentic fatty acid methyl esters and for unavailable methyl esters by the 'semilog' and 'separation factor' procedures (Table 3-3), are included for comparison with the RRt of the components of the samples.

** Values taken from Table 3-3.

tr - Trace component

Table 3-13

RRt	Fatty Acid	Predicted RRt *	Composition (wt %)
1.00	16:0	1.00	8.24 ± 0.14
1.16	16:1w9	1.14	3.36 ± 0.07
1.35	17:0	1.37	0.77 ± 0.01
1.55	17:1w9 (iso 16:0)	1.51	2.24 ± 0.26
1.63	16:4w6	1.67	-
1.72	17:2w4	1.73	tr
1.83	18:0	1.82	4.68 ± 0.12
2.06	18:1w9	2.04	17.16 ± 0.15
2.34	18:2w9	2.34	-
2.46	18:2w6	2.44	3.17 ± 0.02
2.80	18:3w6	2.80	0.41 ± 0.00
3.09	18:3w3	3.13	0.91 ± 0.02
3.43	18:4w3	3.58	0.25 ± 0.01
3.75	20:1w9	3.68	1.44 ± 0.01
4.23	20:2w9	4.22	0.09 ± 0.00
4.50	20:2w6	4.46	1.78 ± 0.01
5.08	20:3w6	5.02	0.23 ± 0.04
5.54	20:4w6) 20:3w3	5.52	14.03 ± 0.10
6.17	20:5w6	6.12	0.39 ± 0.03
7.04	20:5w3	7.04	33.49 ± 0.26
7.68	22:2w9	7.63	0.11 ± 0.01
8.24	22:2w6	7.96	0.09 ± 0.00
9.31	22:3w6	9.15	tr
9.83	22:4w9	9.70	0.61 ± 0.03
10.48	22:5w9	10.51	0.10 ± 0.03
11.27	22:5w6	11.06	0.50 ± 0.01
12.89	22:5w3	12.76	1.44 ± 0.04
14.18	22:6w3	14.19	4.30 ± 0.10
-	Others	-	0.24 ± 0.05

Table 3-14: The carbon chain length (a) and the fatty acid unsaturation (b) class distribution of the total phospholipid fraction from the muscle of 4°C acclimated crayfish, maintained under long and short daylength conditions

* Values reported are the % total weight (= peak area) for each class and are the average ± S.E.M. of replicate analyses (see Table 3-13).

Table 3-14a

Carbon Chain Length	16	17	18	20	22	Others
4°C Short Daylength (n=5)	11.61 ± 0.18* (11.06-12.13)	3.02 ± 0.26 (2.41-3.83)	26.58 ± 0.19 (26.12-27.30)	51.44 ± 0.38 (50.42-52.30)	7.16 ± 0.21 (6.77-7.83)	0.38 ± 0.09 (0.17-0.76)
4°C Long Daylength (n=5)	13.90 ± 0.22 (13.44-14.51)	2.04 ± 0.38 (0.72-3.04)	29.74 ± 0.18 (29.42-30.42)	46.00 ± 0.40 (44.68-46.83)	7.04 ± 0.12 (6.79-7.40)	1.20 ± 0.28 (0.5 - 2.11)
t	8.056	2.128	12.074	9.860	0.496	2.788
P	$\ll$0.001	0.05=0.1	$\ll$0.001	$\ll$0.001	0.6-0.7	0.02-0.05

Table 3-14b

Number of Olefinic Bonds	0	1	2	3	4	5	6	Others
4°C Short Daylength (n=5)	13.69 ± 0.21* (13.40–14.52)	24.20 ± 0.37 (23.27–25.09)	5.25 ± 0.04 (5.13–5.34)	1.50 ± 0.03 (1.45–1.60)	14.90 ± 0.13 (14.56–15.29)	35.91 ± 0.08 (35.09–36.55)	4.30 ± 0.10 (4.07–4.61)	0.38 ± 0.09 (0.17–0.70)
4°C Long Daylength (n=5)	14.99 ± 0.23 (14.61–15.61)	25.87 ± 0.33 (24.79–26.60)	6.29 ± 0.07 (6.14–6.54)	2.05 ± 0.05 (1.85–2.27)	11.21 ± 0.19 (10.53–11.62)	33.99 ± 0.24 (33.19–34.39)	4.33 ± 0.04 (4.20–4.45)	1.28 ± 0.31 (0.51–2.13)
t	4.174	3.368	12.900	6.437	16.028	7.589	0.279	2.788
P	0.001–0.01	0.001–0.01	$\ll$0.001	$<$0.001	$\ll$0.001	$<$0.001	0.7–0.8	0.02–0.05

Table 6-15: Summary of Analyses of the Effect of Thermal Acclimation upon Lipid Composition

Organism	Tissue	Reference
MICROORGANISMS		
Bacillus cereus	Whole Cell	Kaneda (1972)
E. coli	Whole Cell	Aikara, Kato, Ishinaga and Kito(1972
E. coli	Whole Cell	Silbert, Ladenson and Honegger (1973
E. coli	Whole Cell	Haest, De Gier and Van Deenen (1969)
Serratia	Whole Cell	Bishop and Still (1963)
Candida	Culture	Kates and Baxter
Anabaena	Whole Cell	Holton, Blecker and Onore (1964)
Pseudomonas	Whole Cell	Cullen, Phillips and Shipley (1971)
INVERTEBRATES		
Astacus	H.P. Carcass	Broertjes and Zandee (1969)
Musca	Whole Animal	Robb, Hammond and Breber (1972)
Periplaneta	Fat Body	Jamil and Naidu (1968)
Calliphora	Whole Animal)	Fraenkel and Hopf (1940)
Phormia	Whole Animal)	
Blatta	Whole Animal	Munson (1953)
VERTEBRATES		
Rana	Liver Muscle	Baranska and Wlodawer (1966)
Carassius	Brain	Johnston and Roots (1964) Roots and Johnston (1968) Roots (1968)
	Intestine	Kemp and Smith (1970)
Various fish	Mitochondria	Hoar and Cottle (1952) Caldwell and Vernberg (1970) Knipprath and Meads (1965, 1966, 1968)
Hamster	Depot Fat	Fawcett and Lymon (1954)
Rat	Liver)	Williams and Platner
Hamster	Adipose)	

Legend H.P. - Hepatopancreas

Figure 3-1: The Effect of Carrier Gas Flowrate upon the Column Efficiency and Resolution of the Chromatographic Column

Methods

At each carrier gas flowrate the authentic fatty acid mixture 189-1 was analysed.

Column efficiency was calculated for methyl stearate, and peak resolution for the separation of stearate (18:0) and oleate (18:1).

Carrier gas flowrates were measured using a bubble flowmeter.

Legend

Ordinate - Column Efficiency (Theoretical plates), Resolution

Abscissa - Carrier gas flowrate (ml/min).

● - Values of Column Efficiency

O - Values of Peak Resolution

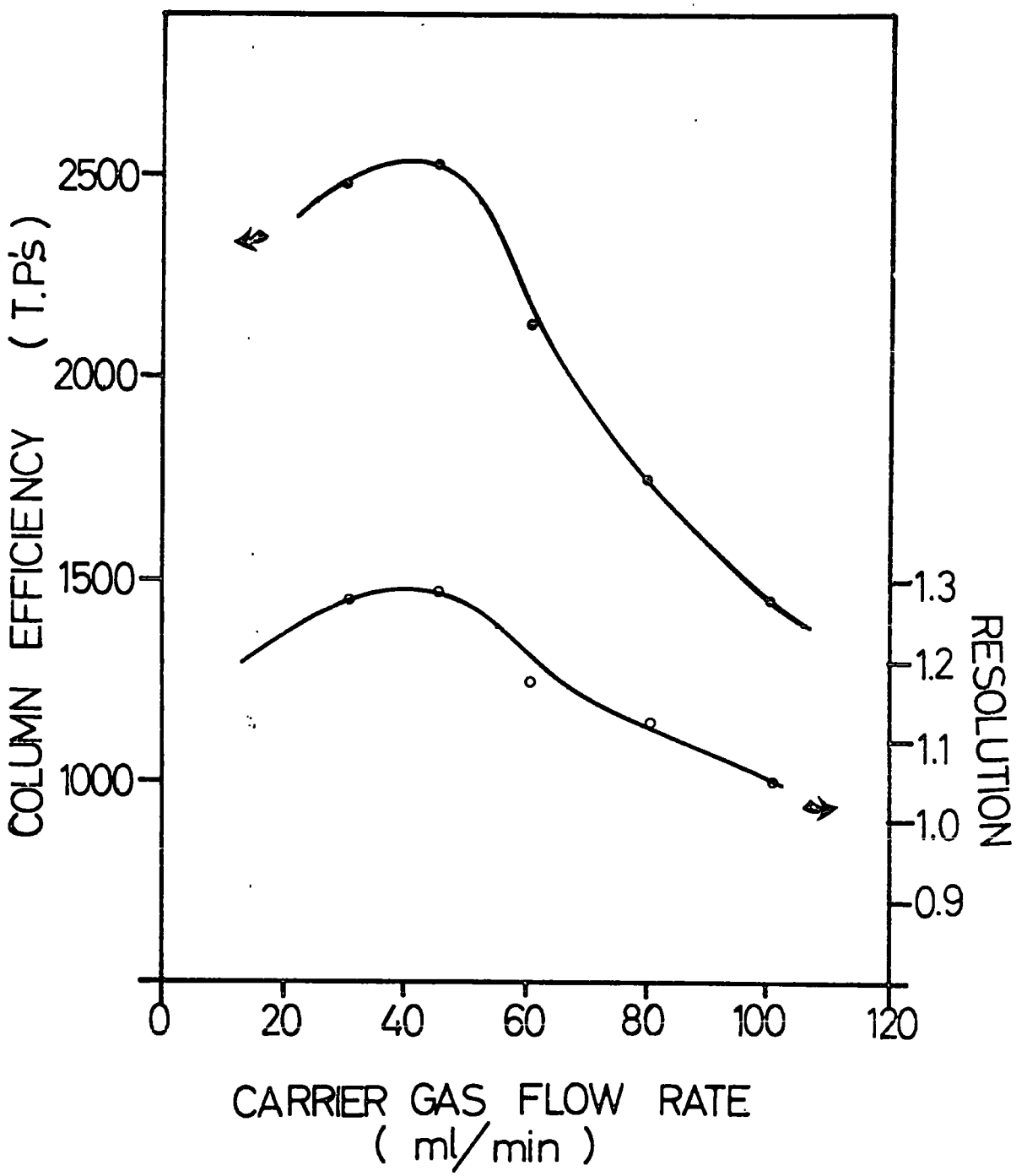

COLUMN EFFICIENCY (T.P's)
2500
2000
1500
1000
1.3
1.2
1.1
1.0
0.9
RESOLUTION
0
20
40
60
80
100
120
CARRIER GAS FLOW RATE
(ml/min)

Figure 3-2: Calibration Line relating the maximal response of the Flame Ionisation Detector to the mass of the sample applied

Method

8ul of a solution containing various concentrations of methyl palmitate (16:0) in carbon disulphide was injected. The height of the chromatographic peak was measured and the maximal detector response determined as described in 'Methods'.

Legend

Ordinate - Maximal Detector Response (Amps)

Abscissa - Load of methyl palmitate (gms)

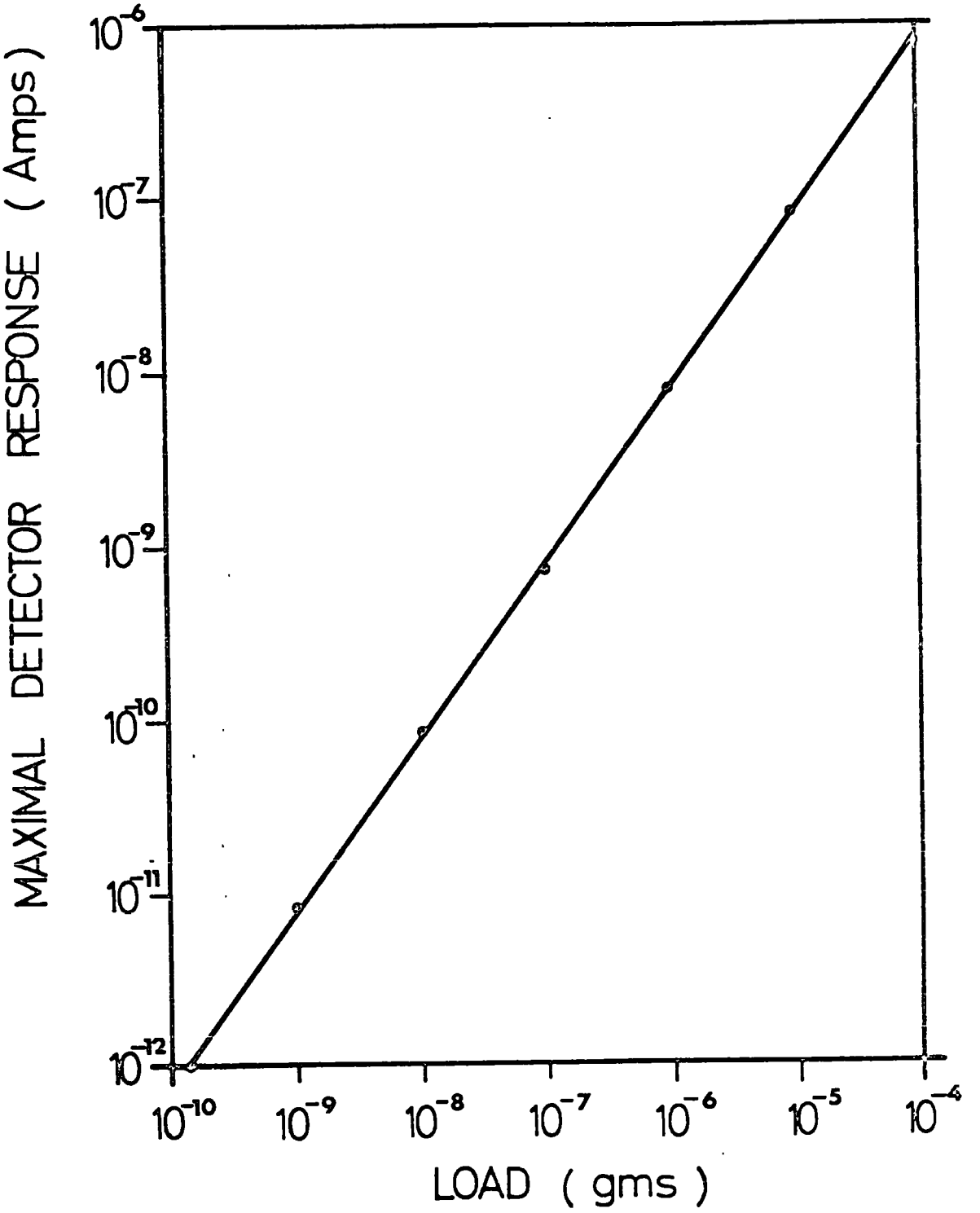

MAXIMAL DETECTOR RESPONSE (Amps)
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
10^{-11}
10^{-12}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
LOAD (gms)

Figure 3-3: The Semilog plots for authentic fatty acid methyl esters and for unavailable methyl esters given by Burchfield and Storrs (1962) and Ackman (1963a, b).

Methods

The RRt (16:0) for each methyl ester was determined by chromatography of the authentic sample, or from the literature. The values for the monoenoic fatty acids were plotted first, and in accordance with the system of Ackman (1963a), lines for other fatty acid families were drawn parallel to this. RRt for unavailable fatty acids was predicted by interpolation on the lines.

The lines for the mono-, tri-, tetra-, penta- and hexaenoic fatty acid methyl esters are illustrated in (a), and those for monoenoic and dienoic fatty acids in (b).

Legend

Ordinate - Relative Retention Time (16:0)

Abscissa - Carbon Chain Length

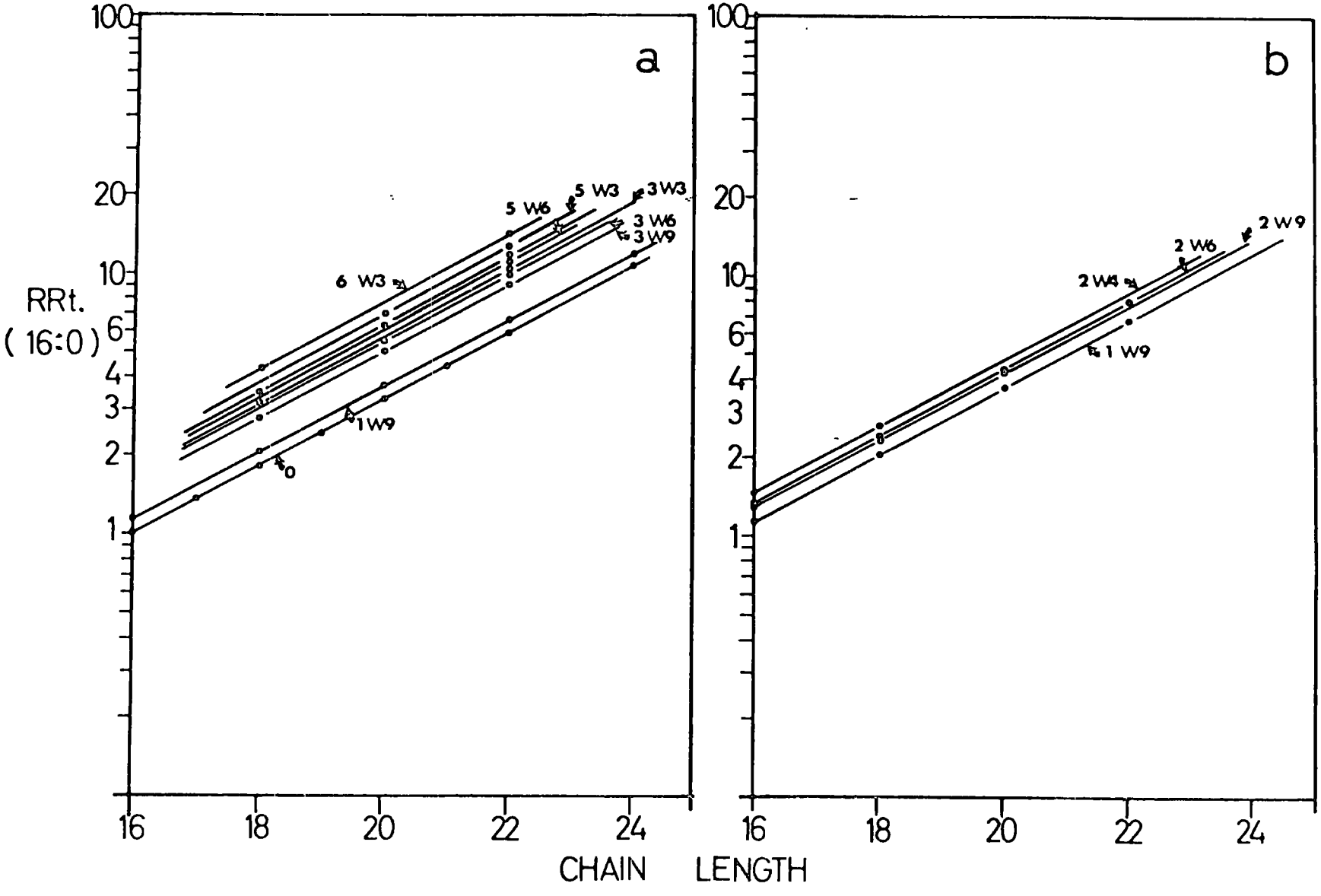

a
b
RRt.
(16:0)
100
20
10
6
4
3
2
1
16
18
20
22
24
CHAIN LENGTH
6 W3
5 W6
5 W3
3 W3
3 W6
3 W9
1 W9
0
2 W6
2 W9
1 W9

Figure 3-4: Gas-Liquid Chromatograms of the Fatty Acid Methyl Ester Fractions separated by 'Argentation' Chromatography

Methods

The fatty acid methyl esters of purified choline phospholipids from crayfish muscle were fractioned by TLC on silver nitrate impregnated chromatoplates as described in 'Methods'. A gas-liquid chromatographic analysis of each fraction is presented - together with the tentative identity of the major peaks.

FRACTION 1.
18:1 w9
18:0
15:1 w9
15:0
FRACTION 2.
20:5 w3
20:4 w6
20:2 w6
18:2 w6
FRACTION 3.
22:3 w6
20:3 w3
18:3 w3
FRACTION 4.
22:4 w6
20:4 w3
18:4 w6
16:4 w6
FRACTION 5.
22:6 w3
22:5 w3
22:5 w6
22:4 w9
RESPONSE
90
75
60
45
30
15
TIME(mins.)
SOLVENT FRONT

Figure 3-5: Typical Gas-Liquid Chromatogram for the methyl esters of the total phospholipid fraction from crayfish muscle

Methods

See 'Materials and Methods'.

A recording at increased sensitivity is also presented for the latter part of the analysis, together with the tentative identities of the major peaks.

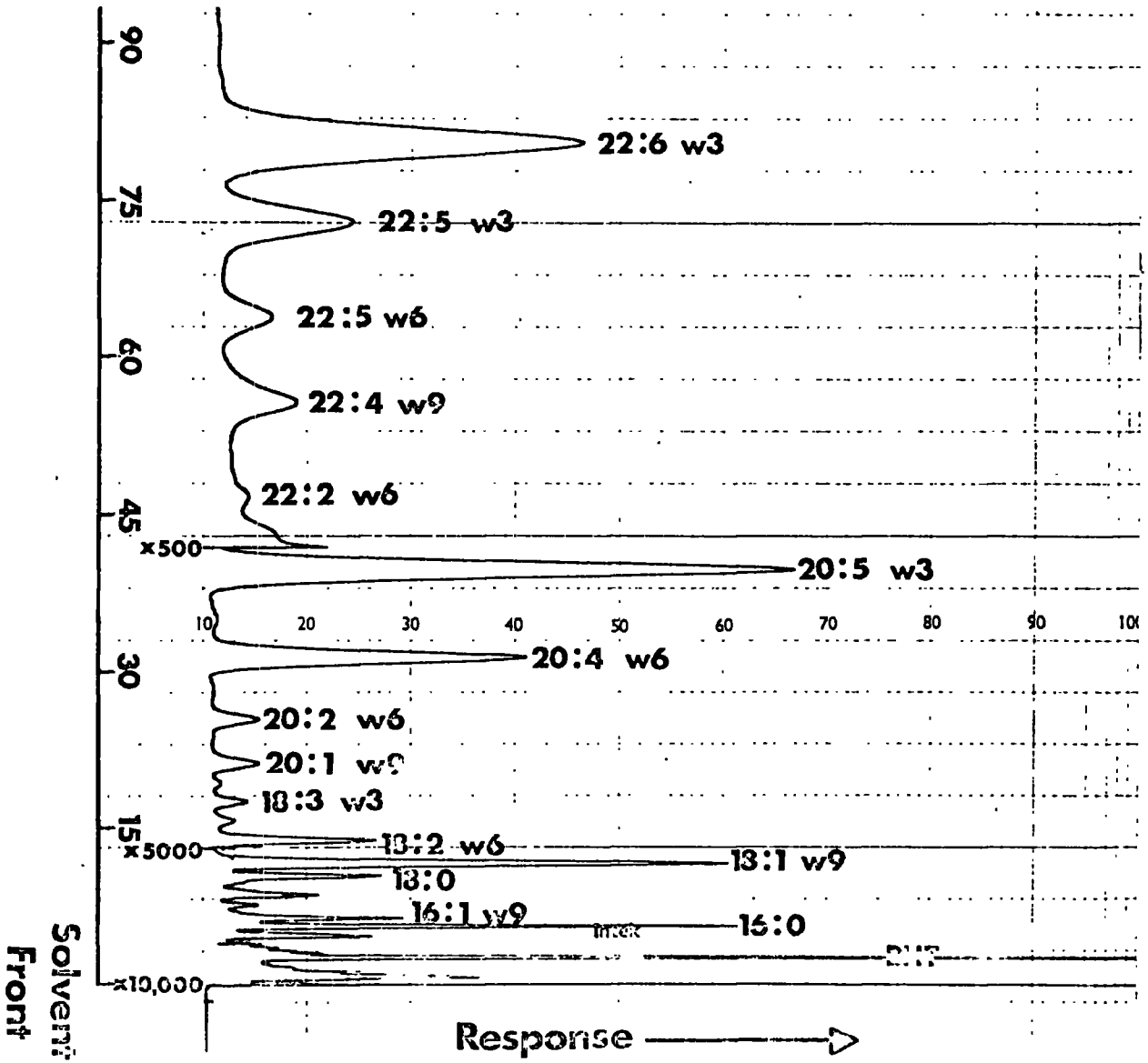

90
75
60
45
30
15
22:6 w3
22:5 w3
22:5 w6
22:4 w9
22:2 w6
×500
20:5 w3
10
20
30
40
50
60
70
80
90
20:4 w6
20:2 w6
20:1 w9
18:3 w3
×5000
18:2 w6
13:1 w9
13:0
16:1 w9
Index
15:0
×10,000
Solvent Front
Response

Figure 3-6: A Histogram of the fatty acid distribution of the total phospholipid class from the lipid extracts of 4°C and 25°C acclimated crayfish

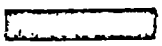
Methods

Data is presented in Table 3-5

Legend

Ordinate - % weight

Abscissa - Fatty Acid Species

 - 4°C Acclimated Crayfish

 - 25°C Acclimated Crayfish

%wt
30
20
10
0
16:0
16:1 ω9
17:0
17:1 ω9
18:0
18:1 ω9
18:2 ω6
18:3 ω6
18:3 ω3
20:1 ω9
20:2 ω6
20:4 ω6
20:5 ω3
22:4 ω9
22:5 ω6
22:5 ω3
22:6 ω3

Figure 3-7: Histograms to illustrate The carbon chain length distribution of the fatty acids of different phospholipid fractions of the lipid extracts of 4°C and 25°C acclimated crayfish

Legend:

a - Total phospholipid fraction

b - Choline phosphoglyceride fraction (PC)

c - Ethanolamine phosphoglyceride fraction (PE)

d - Serine/Inositol phosphoglyceride fraction (PS/PI)

● - Significant differences between classes (see Tables 3-6a, 3-8a, 3-10a and 3-12a)

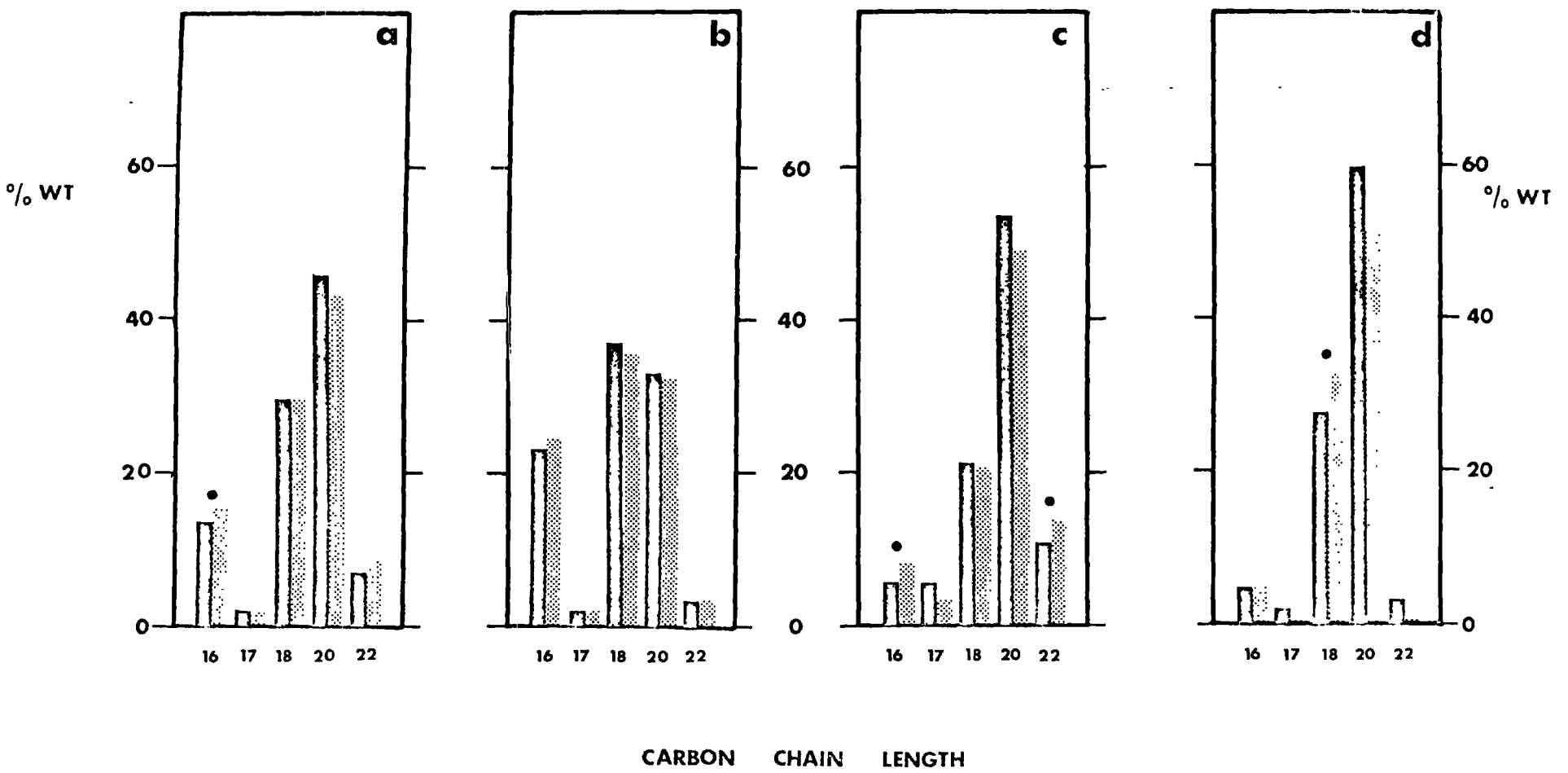

a
b
c
d
% WT
% WT
60
40
20
0
16 17 18 20 22
CARBON CHAIN LENGTH

Figure 3-8: Histograms to illustrate The distribution of unsaturated fatty acid classes of different phospholipid fractions of the lipid extracts of 4°C and 25°C acclimated crayfish

Legend:

a - Total phospholipid fraction

b - Choline phosphoglyceride fraction (PC)

c - Ethanolamine phosphoglyceride fraction (PE)

d - Serine/Inositol phosphoglyceride fraction (PS/PI)

● - Significant differences between acclimation groups (see Tables 3-6b, 3-8b, 3-10b and 3-12b)

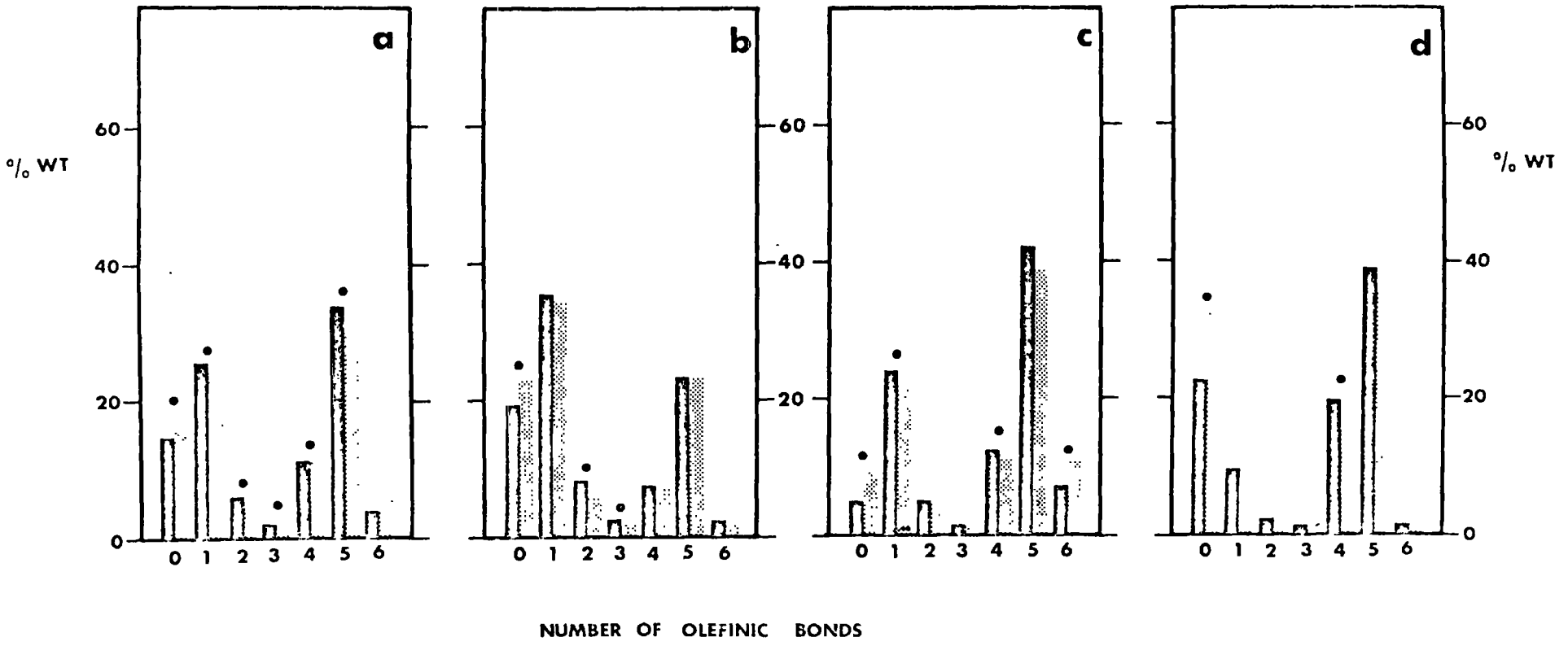

a
b
c
d
% WT
% WT
60
40
20
0
0 1 2 3 4 5 6
NUMBER OF OLEFINIC BONDS

Figure 3-9: Typical Gas-Liquid Chromatogram for the methyl esters of purified choline phospholipids of crayfish muscle

Methods

See 'Materials and Methods'.

A recording at increased sensitivity is also presented for the latter part of the analysis, together with the tentative identities of the major peaks.

RESPONSE
SOLVENT FRONT
×10,000
×2000
BHT
16:0
18:1 w9
16:1 w9
18:0
18:2 w6
18:3 w3
20:1 w9
22:2 w6
20:4 w6
20:5 w3
22:2 w6
22:5 w6
22:5 w3
22:6 w3
TIME(mins.)
15
30
45
60
75
90
105
%

Figure 3-10: A Histogram of the fatty acid distribution of the purified choline phosphoglyceride class from the lipid extracts of 4°C and 25°C acclimated crayfish

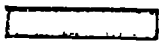
Methods

Data is presented in Table 3-7.

Legend

Ordinate - % weight

Abscissa - Fatty Acid Species

 - 4°C Acclimated Crayfish

 - 25°C Acclimated Crayfish

%wt
30
20
10
0
16:0
16:1ω9
17:0
17:1ω9
18:0
18:1ω9
18:2ω6
18:3ω
18:3ω3
20:1ω9
20:2ω6
20:4ω6
20:5ω3
22:4ω9
22:5ω6
22:5ω3
22:6ω3

Figure 3-11: Typical Gas-Liquid Chromatogram for the methyl esters of purified ethanolamine phospholipids of crayfish muscle

Methods

See 'Materials and Methods".

A recording at increased sensitivity is also presented for the latter part of the analysis, together with the tentative identities of the major peaks.

RESPONSE
SOLVENT FRONT
TIME(mins.)
BHT
16:0
18:0
18:1 w9
18:2 w6
20:1 w9
22:2 w6
20:4 w6
20:5 w3
22:2 w6
22:4 w9
22:5 w6
22:5 w3
22:6 w3

Figure 3-12: A Histogram of the fatty acid distribution of the purified ethanolamine phosphoglyceride class from the lipid extracts of 4°C and 25°C acclimated crayfish

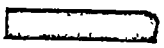
Methods

Data is presented in Table 3-9

Legend

Ordinate - % weight

Abscissa - Fatty Acid Species

 - 4°C acclimated crayfish

 - 25°C acclimated crayfish

%wt
0
10
20
30
16:0
16:1ω9
17:1ω9
17:2ω4
18:0
18:1ω9
18:2ω6
18:3ω3
20:1ω9
20:2ω6
20:4ω6
20:5ω3
22:2ω6
22:4ω9
22:5ω6
22:5ω3
20:6ω3

Figure 3-13: Typical Gas-Liquid Chromatogram for the methyl esters of purified serine/inositol phospholipids of crayfish muscle

Methods

See 'Materials and Methods'.

A recording at increased sensitivity is also presented for the latter part of the analysis, together with the tentative identities of the major peaks.

RESPONSE
SOLVENT FRONT
TIME (mins.)
15
30
45
60
75
90
x5000
x2000
BHT
16:0
18:0
18:1 w9
20:4 w6
20:5 w3
22:6 w3
22:5 w3
22:5 w6
Intelk
%
10
20
30
40
50
60
70
80
90

Figure 3-14: A Histogram of the fatty acid distribution of the purified serine/inositol phosphoglyceride class from the lipid extracts of 4°C and 25°C acclimated crayfish

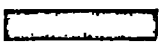
Methods

Data is presented in Table 3-9

Legend

Ordinate - % weight

Abscissa - Fatty Acid Species

 - 4°C acclimated crayfish

 - 25°C acclimated crayfish

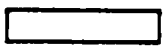
%Wt
30
20
10
0
16:0
16:1ω9
17:0
18:0
18:1ω9
18:2ω6
18:3ω6
18:4ω3
20:1ω9
20:2ω6
20:3ω6
20:4ω6
20:5ω3
22:4ω6
22:5ω6
22:5ω3
22:6ω3

Figure 3-15: The fatty acid distribution of the total phospholipid class from the lipid extracts of 4°C and 25°C, long-daylength acclimated crayfish, and 4°C, short-daylength acclimated crayfish

Legend

Ordinate - % weight

Abscissa - Fatty Acid class

 - 4°C, 8 hour-light photoperiod acclimated crayfish

 - 4°C, 18 hour-light photoperiod acclimated crayfish

 - °C, 18 hour-light photoperiod acclimated crayfish

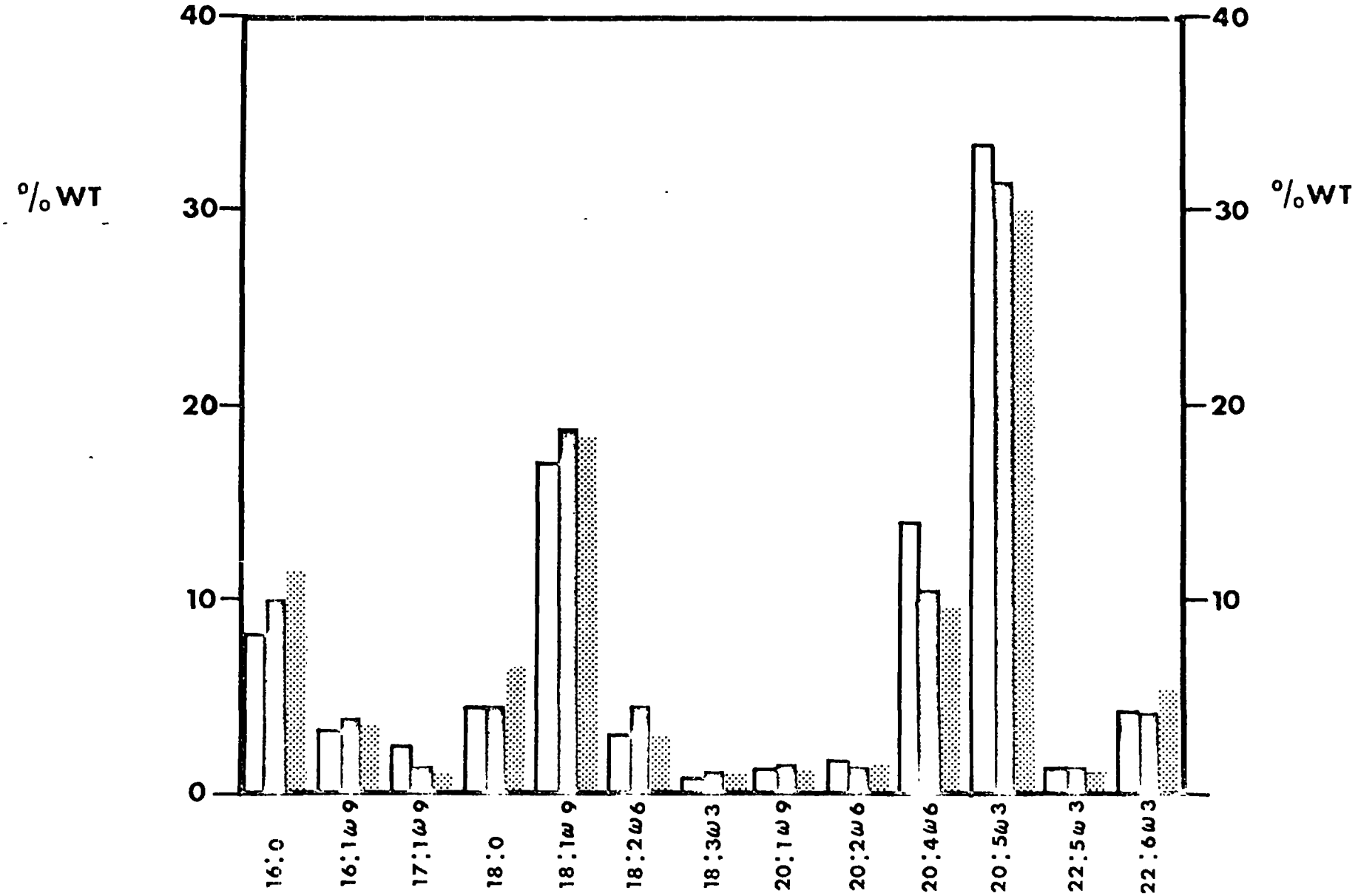

%WT
40
30
20
10
0
%WT
16:0
16:1ω9
17:1ω9
18:0
18:1ω9
18:2ω6
18:3ω3
20:1ω9
20:2ω6
20:4ω6
20:5ω3
22:5ω3
22:6ω3

Figure 3-16: The carbon chain length (a) and the fatty acid unsaturation class distribution (b) of 4°C acclimated crayfish maintained under long and short photoperiod

Legend

Ordinate - % weight

Abscissa - Carbon chain length (a)

Number of olefinic bonds (b)

o - Significant differences (see Table 3-14) between classes.

- 4°C, 8 hour-light photoperiod acclimated crayfish

- 4°C, 18 hour-light photoperiod acclimated crayfish

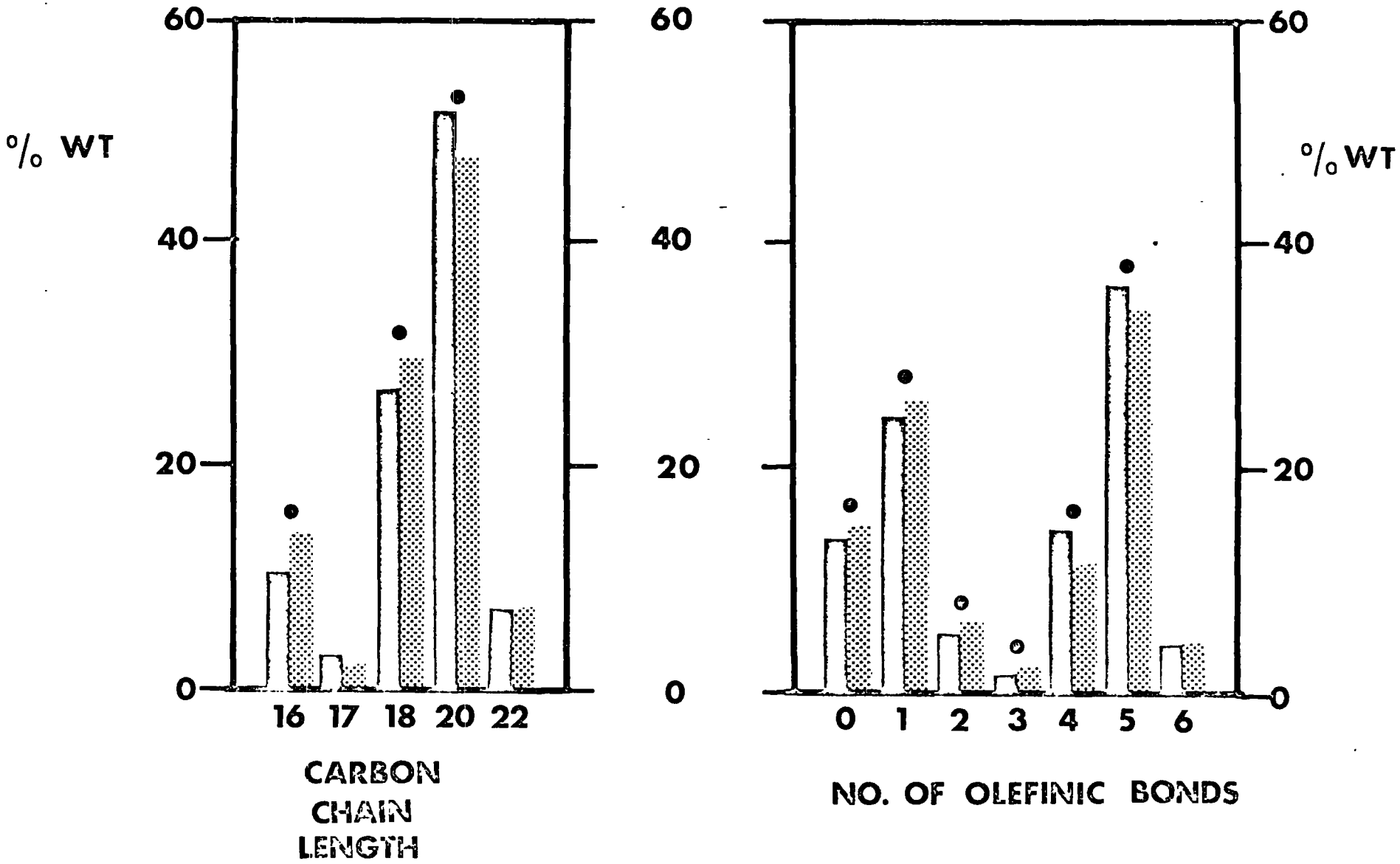
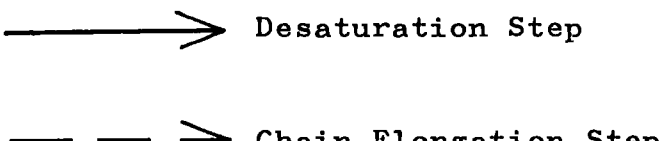

% WT
60
40
20
0
16 17 18 20 22
CARBON CHAIN LENGTH
60
40
20
0
% WT
60
40
20
0
0 1 2 3 4 5 6
NO. OF OLEFINIC BONDS

Figure 3-17: The Proposed Biosynthetic Pathways for the Production of the Polyunsaturated Fatty Acids Identified in Crayfish Muscle, from the Dietary Oleic, Linoleic and Linolenic fatty acids (see Gurr and James, 1971).

Legend



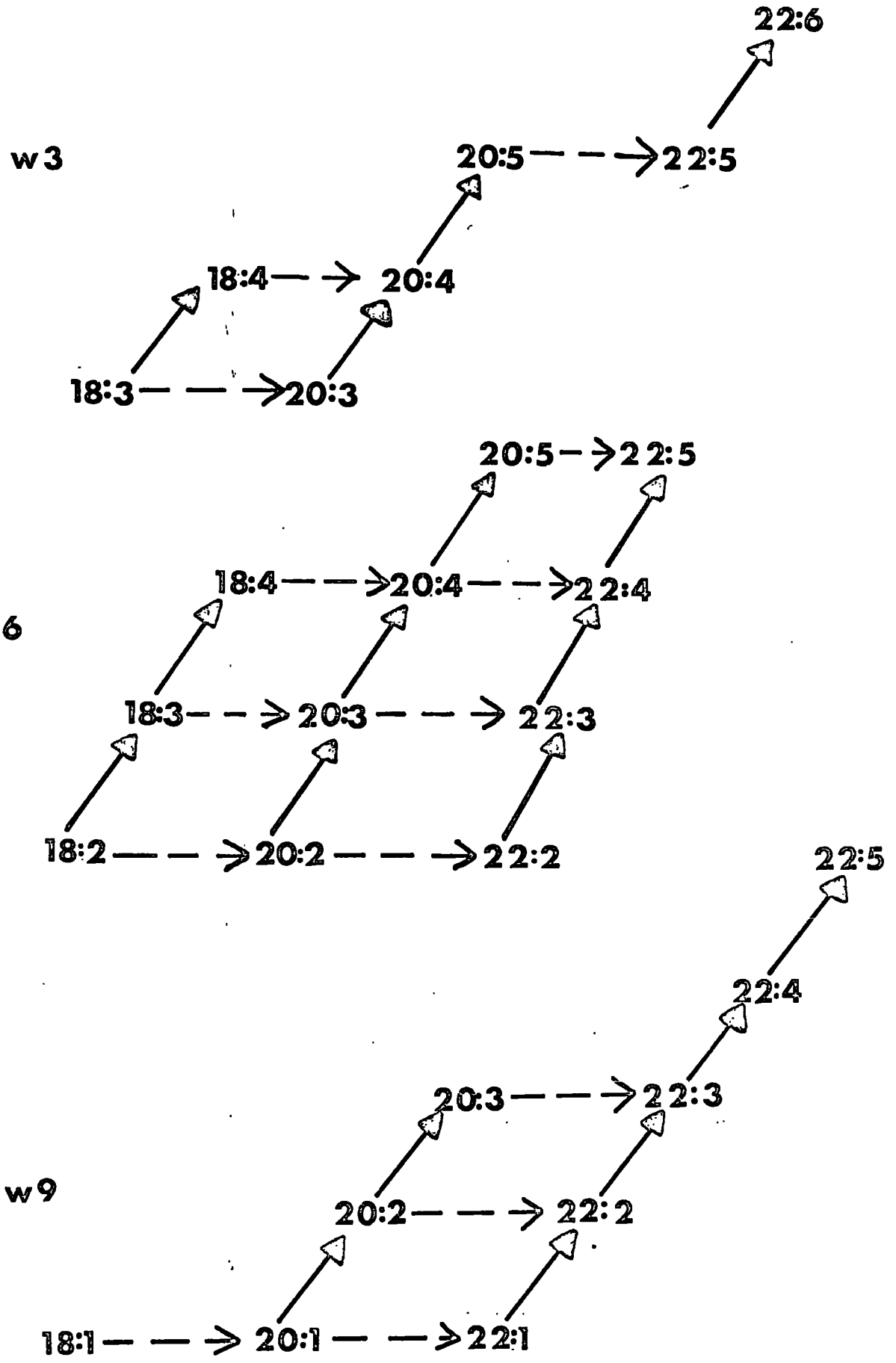

22:6
ω3
20:5
22:5
18:4
20:4
18:3
20:3
20:5
22:5
18:4
20:4
22:4
ω6
18:3
20:3
22:3
18:2
20:2
22:2
22:5
22:4
20:3
22:3
ω9
20:2
22:2
18:1
20:1
22:1

Figure 3-18: The effect of thermal acclimation under short and long daylength conditions upon the thermal resistance of the freshwater crayfish

Methods

Freshly trapped crayfish were acclimated under their respective conditions for 21 days. Thermal resistance was measured using the technique of Bowler (1963a) in clean aerated tapwater at a temperature of 32°C. The criterion of heat death was the complete cessation of all movements (Bowler, 1963a). The results for 25°C, 18 hour-daylength acclimated crayfish are shown in (a) and the results for both 4°C, 8 hour and 18 hour-daylength acclimated crayfish in (b).

The results were plotted as % survival as a function of time at 32°C.

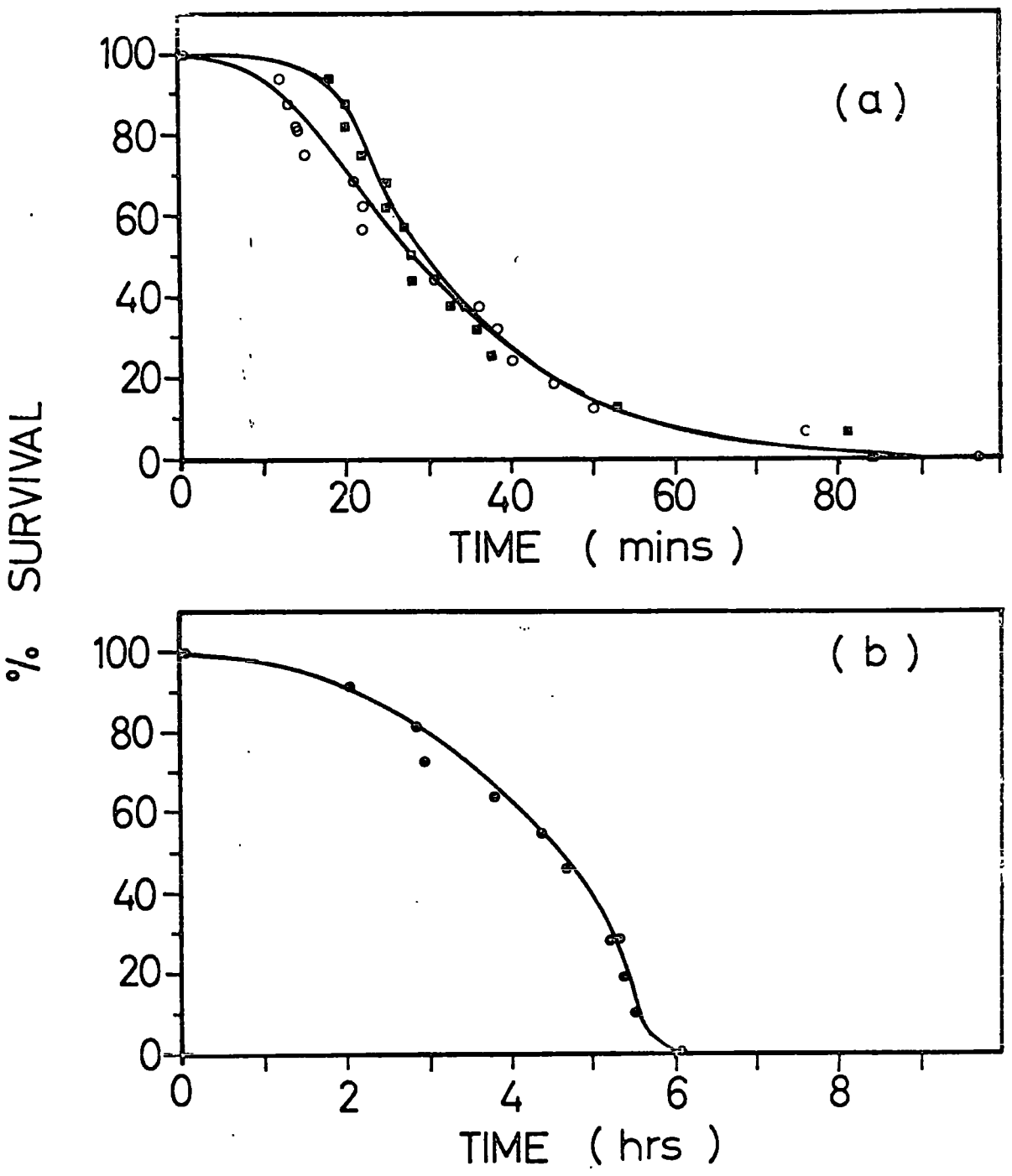
Legend

Ordinate - % survival

Abscissa - Time at 32°C

Acclimation treatment

- O 4°C, 8 hour photoperiod
- ◘ 4°C, 18 hour "
- ◘ 25°C, 18 hour "



Section II

STUDIES ON THE ACTIVITY OF A MEMBRANE-BOUND ENZYME

" . . . nature has learned so to exploit the biochemical situation as to escape the single application of the Arrhenius equation . . ."

Barcroft, J. (1934) Features in the Architecture of Physiological Function. Cambridge University Press, London, p.40.

Chapter 4

THE ROLE OF MEMBRANE-ENZYMES IN TEMPERATURE COMPENSATION

Thermal compensations are usually manifest as a rate change in some physiological function, such as oxygen consumption in Lumbriculus sp. (Kirberger 1953) and Pachygrapsus sp. (Roberts 1957), development of Rana pipiens (Ryan 1941) and heart-beat rate in Gammarus sp. (Krog 1954), or as some change in the resistance to lethal temperatures, as observed in the freshwater crayfish, Austropotomobius sp. by Bowler (1963). For a general review see Prosser (1973) and Precht, Christophersen, Hensen and Larcher (1973). The growth of biochemical knowledge and the development of more penetrative analytical techniques since the 1950's has led to numerous studies to explain such physiological compensatory responses to temperature in terms of cellular, subcellular and molecular phenomena. That enzymes and enzyme systems show compensatory responses has been known since the pioneering work of Precht and his colleagues. They found that homogenates of isolated tissues, from a variety of invertebrates and microorganisms, showed significantly greater oxidase activity, both in the presence and absence of added substrate, when taken from individuals acclimated to low temperatures than in samples from warm-acclimated organisms (Precht, Christophersen and Hensel, 1955).

Until recently, mechanistic interpretations of these effects have been based largely on changes in the cellular concentration of the enzyme concerned. This has been claimed by Eckberg (1962) in crucian carp gills, by Baslow and Nigrelli (1964) in brain cholinesterases of the killifish, and by Freed (1965) on the phosphofructokinase of variously acclimated goldfish. In these studies it was usually assumed that the concentration of an enzyme was proportional to its

activity at saturating substrate concentrations. Whatever the means of modifying cellular enzyme concentrations (i.e., by changes in the rates of enzyme synthesis or degradation) this approach has obvious limitations. Firstly, the solvent capacity of a cell is strictly finite, as are the availability of binding sites for membrane-bound enzymes. Thus it is inconceivable that all enzymes, or even all rate-limiting ones compensate by an increase in the concentration of active molecules during cold acclimation. Secondly, the catalytic and regulatory properties of enzymes are also usually temperature sensitive, so an unmodified enzyme may prove markedly inefficient at the new environmental temperature (see Hochachka and Somero 1973). Thirdly, enzymes may prove markedly thermolabile under the new thermal regime. Fourthly, and perhaps most important, the physiological relevance of enzyme activity measurements at saturating levels of substrate is questionable, since this parameter gives no indication of enzyme behaviour at the substrate concentrations presumed to exist intracellularly. (Freed,1965; Hochachka and Somero,1973). At present, there is no reliable technique for measuring the in vivo concentration of enzymes. Thus whilst it is not possible to exclude changes in enzyme concentration from participating in compensatory processes it seems likely that other phenomena are involved.

The introduction of the concept of allosteric modulation of enzyme activity and also the recognition that temperature per se has profound effects upon the kinetic and regulatory characteristics of enzymes (Hochachka and Somero, 1973), has greatly increased our understanding of the effect of temperature on enzyme function. This has led to the

realisation that adaptational changes in enzyme activity may be mediated by a host of subtle and hitherto unsuspected mechanisms not related to changes in enzyme concentration. Feeny, Vandenheede and Osuga (1972) have listed some possible differences between the termperature characteristics of enzymes from warm- and cold- adapted organisms (see figure 4-1):

1. An enzyme from cold-adapted organisms may be more labile at high temperatures than the same enzyme from warm-adapted organisms.
2. An enzyme from cold-adapted organisms may have a higher specific activity at all temperatures, but the same Arrhenius activation energy, as the same enzyme from warm-adapted organisms.
3. An enzyme from cold-adapted organisms may have a lower activation energy than the same enzyme from warm-adapted organisms.
4. The enzyme from warm-adapted organisms may have a termperature-induced transition in activity.
5. The enzyme from cold-adapted organisms may have minimal Km (maximum enzyme-substrate affinity) at low temperatures, and the enzyme from warm-adapted organisms a minimal Km at higher temperatures.
6. The enzymes from warm-adapted organisms may be allosterically inhibited at low temperatures.

These potential mechanisms may result in the total or partial compensation for the decreased rates of enzyme activity and metabolism generally, that occur at lower environmental temperature in poikilotherms. This may permit some degree of physiological independence from environmental conditions. The potential adaptational mechanisms outlined above are not mutually exclusive, and compensatory

phenomena are likely to be the result of a variety of changes, all contributing to a higher enzyme activity or metabolic rate shown by cold-acclimated organisms. In addition, it should be understood that apart from the general effects of thermal energy upon the molecular motion of molecules, temperature may have both direct and indirect effects upon enzyme activity. For example, cellular temperature may directly cause conformational changes in enzyme structure which modify its kinetic and/or regulatory properties. Alternatively, a change in cell temperature may indirectly affect enzyme function by causing changes in cellular pH or cation content.

Hochachka and Somero (1973) have suggested that these enzymic adaptations may be brought about in three ways. First, there may be quantitative changes in the number of active enzymes. Second, there may be qualitative changes in the types of enzymes present. Third, there may be modulation of the kinetic and/or regulatory properties of existing enzymes. These mechanisms of adaptation have been termed the "Quantitative", "Qualitative" and "Modulative" strategies of adaptation respectively (Hochachka and Somero, 1973).

In view of the implication of muscle membrane phenomena in the heat death of crayfish (Bowler 1963, Gladwell 1973, Bowler, Duncan, Gladwell and Davison, 1973) and the effect of temperature acclimation upon heat death (Bowler 1963, Gladwell 1973) and muscle lipid composition (see Section I), it was of interest to know whether the enzyme component of muscle membranes showed any kinetic or regulatory adaptations in response to maintenance of the animal at different temperatures. The effect of a changed phospholipid environment on membrane-bound

enzymes was an obvious possible mechanism for modulation of their catalytic properties. This latter suggestion is supported by the observation by Bloj, Morero, Farias and Trucco (1973) that membrane fluidity has a marked effect upon the allosteric behaviour of the Na^+-K^+-ATPase and Acetylcholinesterase of rat erythrocyte membranes (see also Sineriz, Bloj, Farias and Trucco 1973).

The Calcium-stimulated ATPase (E.C. 3.6.1.3.) associated with the sarcoplasmic reticulum of striated muscle proved to be a particularly convenient 'model' system with which to study the effects of temperature acclimation. The sarcoplasmic reticulum is a membranous vesicle, specialised for the sequestration of calcium from the sarcoplasm, causing relaxation of the contractile machinery (for review see Ebashi and Endo, 1968). Its primary advantage is that it contains few protein species other than the Ca^{2+}-activated ATPase which may constitute up to 80-90% of microsomal protein (Deamer 1973, Meissner and Fleischer 1971, Inesi, Blanchet and Williams 1972, Madeira, Antunes - Madeira and Carvalho 1974). Indeed, it has been claimed that there is no other major enzymic activity associated with the vesicles, although several minor enzyme activities such as hexokinase have been detected (Martonosi 1972).

The ATPase is intimately bound to the membrane and is highly dependent upon it for its activity. In contrast to most other transport systems, both the catalytic function (i.e., ATPase) and transport activity (uptake of calcium) can be monitored easily. An additional asset is its availability in relatively large amounts, from striated muscle. Macruran abdominal muscle, generally, is notable both for its well developed sarcoplasmic

reticulum and scarcity of mitochondria (Van der Kloot, 1969; Baskin, 1971; Deamer, 1973). This results in a relatively pure preparation, with few contaminating membranes and a high specific activity (Deamer, 1973).

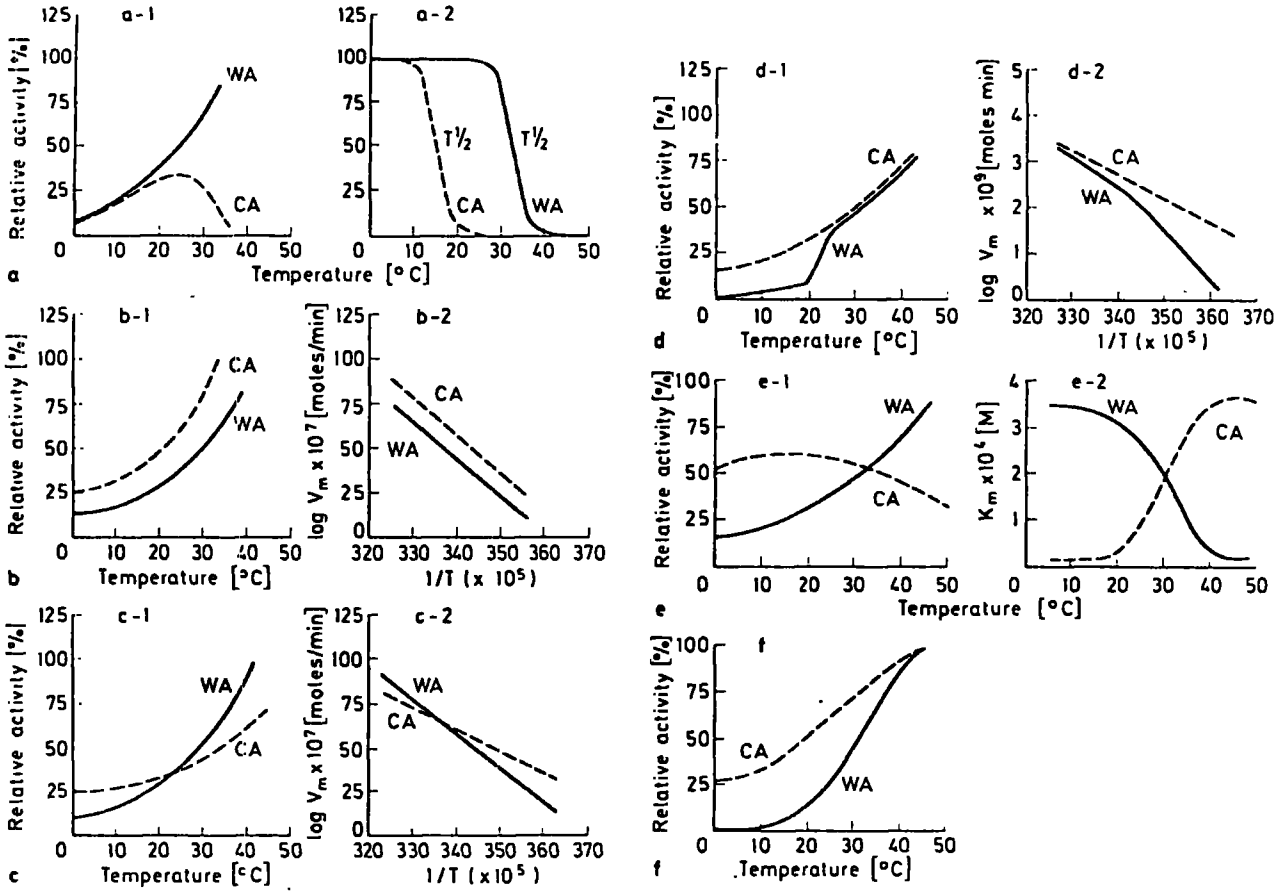


Fig. 4–1 Hypothetical plots of the different ways that cold-adapted and warm-adapted enzymes might be affected by temperature. CA = cold-adapted; WA = warm-adapted.

a Cold-adapted enzyme labile at higher temperature. a-1 Activities at different temperatures. a-2 Loss of activity after incubation for given period of time at indicated temperature.

b Cold-adapted enzyme has higher specific activity at all temperatures but has same activation energy as warm-adapted enzyme. b-1 Activities at different temperatures. b-2 Arrhenius plots.

c Cold-adapted enzyme has lower activation energy than warm-adapted enzyme. c-1 Activities at different temperatures c-2 Arrhenius plots

d Warm-adapted enzyme has temperature induced transition. d-1 Activities at different temperatures. d-2 Arrhenius plots.

e Cold-adapted enzyme has minimal K_m at low temperature. e-1 Activities at different temperatures e-2 Effect of temperature on K_m values

f Warm-adapted enzyme is inhibited allosterically at low temperature. Activities at different temperatures

Chapter 5.

GENERAL MATERIALS AND METHODS

This chapter contains a description of materials and methods common to the experimental work considered in Sections III and IV. The techniques used in specific experiments are given at relevant points in the text.

MATERIALS

a. Glassware Pipettes were supplied by J.A. Jobling and Co. Ltd. (Treforest) and were E-Mil Gold Line Standard (BS 700). Other glassware was supplied by J.A. Jobling and Co. Ltd. (E-Mil Gold Line and Pyrex brands) and A. Gallenkamp Ltd. (Technico).

b. Animals Crayfish (Austropotamobius pallipes) were obtained from reservoirs and streams in Northumberland, and maintained at 15°C and an 18 hour day length in the laboratory as described in Chapter 2.

Groups of animals were acclimated in clean aerated water at 4 ±0.5°C or 25 ±0.1°C for not less than 21 days. All animals were in the intermoult condition when sacrificed.

c. Chemicals $MgCl_2.6H_2O$, KCl, HCl, sucrose and $(NH_4)_6\ Mo_7O_{24}.4H_2O$ were purchased from British Drug House Chemicals Ltd. (Poole, Dorset) in the purest form available ('AnalaR' Grade). Sodium azide (Laboratory Grade) and $CaCl_2$ (AVS volumetric solution) were also supplied by BDH Chemicals Ltd.

Tris (hydroxymethyl) aminomethane (Trizma Base; Primary Standard and Buffer), Bovine Serum Albumin (fraction V, 96-99%),

Disodium-Adenosine Triphosphate (Sigma Grade), Imidazole free base (Grade 1) and Ethyleneglycol-bis (β-amino-ethylether) N, N'-tetraacetic acid (EGTA) were purchased from the Sigma London Chemical Co. Ltd. (Kingston-upon-Thames, Surrey).

Disodium-ATP was converted to its acid form by shaking with Dowex H^+ resin. The H^+-ATP was recovered from the supernatant by centrifugation of the resin. H^+-ATP was converted to its Tris-salt by titration with 2M Tris to pH 7.0, and stored until required at -20°C.

'Dowex' 1-X8 (20-50 U.S. Mesh) was obtained from BDH Chemicals Ltd. 'Quadralene' Laboratory Detergent was purchased from Fisons Scientific Apparatus Ltd. (Loughborough). 'Lubrol W' and 'Cirrasol ALN-F' were a gift from ICI Ltd., Dyestuffs Division.

METHODS

a. Treatment of Glassware All glassware was soaked overnight in a 2% (w/v) solution of 'Quadralene' laboratory detergent, rinsed 6 times in hot tapwater and 6 times in distilled water. Glassware was oven-dried. Glass-Teflon homogenisers and polycarbonate centrifuge tubes were allowed to drain at room temperature.

b. Preparation of Microsomes Crayfish were acclimated to either 4°C or 25°C as described in Chapter 2. The abdominal flexor and extensor muscles were rapidly excised from the animal and placed immediately into 10 volumes (vol: gm wet weight

of muscle) ice-cold extraction medium (100mM KCl, 10mM Imidazole - HCl, pH 7.1) and weighed. All subsequent operations were performed on crushed ice.

The muscle was finely minced in a Vortex Waring Blender (M.S.E. Ltd.) using a stainless steel impellor rotating at approximately 4,000 r.p.m. for 2-3 minutes, and then homogenised with 10 passes of a Potter-Elvehjem homogeniser with a teflon pestle (clearance 0.10 - 0.15 mm) rotating at approximately 2,000 r.p.m. This crude homogenate was centrifuged in a 'Mistral 2L' Centrifuge (M.S.E. Ltd.) at 1,000g and 4°C for 30 minutes to remove nuclei, connective tissue and cell debris. The supernatant (A) was stored on ice, and the pellet re-extracted by homogenisation in 5 volumes of extraction medium followed by centrifugation as before to yield supernatant (B).

Supernatants A and B were combined and spun at 13,000g and 4°C, for 30 minutes in a 'High Speed 18' Refrigerated Centrifuge (M.S.E. Ltd.) to remove remaining nuclei, myofibrils and mitochondria. The supernatant fraction (C) containing microsomes, soluble proteins and myofibrillar contaminants was centrifuged at 35,000g for 60 minutes at 4°C. The microsomal pellet was resuspended in a concentrated KCl medium (0.6M KCl, 10mM Imidazole - HCl, pH 7.1) by homogenisation, in order to solubilise contaminating fragments of actomyosin (Martonosi and Feretos, 1964). The

heavy microsomes were finally sedimented at 35,000g for 60 minutes at 4°C and resuspended by thorough homogenisation in a sucrose medium (0.3M Sucrose, 10mM Imidazole - HCl, pH 7.1) to give a protein concentration of 40-80 ug microsomal protein/ml. (The Ca^{2+}-Mg^{2+}-ATPase was found to be unstable when frozen in 10mM Imidazole - HCl, pH 7.1, alone but stable in the sucrose medium.) The preparation was used immediately or stored at -20°C in the dark.

c. Reaction Media Two ionic reaction media were used:

(i) 3mM $MgCl_2$

(ii) 3mM $MgCl_2$, 0.5mM $CaCl_2$

Both contained 0.5mM EGTA, 100mM KCl, 75mM Sucrose, 25mM Imidazole - HCl, pH 7.1 (all final concentrations). Tris-ATP was added to the reaction medium prior to assay to give a final concentration of 3mM. The Ca^{2+}-stimulated ATPase was defined as the difference between the ATPase activity in presence of $MgCl_2$ and $CaCl_2$, and ATPase activity in the presence of $MgCl_2$ alone.

All solutions were made up in deionised water. Deviations from these conditions and methods are noted at relevant points in the text.

d. ATPase Assay Enzyme activities were assayed either in 150 X 25mm 'Pyrex' boiling tubes, or glass centrifuge tubes. The reaction was usually started by addition of 0.5ml microsomal preparation to 1.5ml of the appropriate assay medium containing Tris-ATP. The latter solution had been equilibrated previously at the desired

incubation temperature. All temperatures of assay were controlled to $\pm 0.1^{\circ}C$ by a 500 watt immersion heater connected by a hotwire vacuum switch relay (Sunvic Controls Ltd.) to a 'Jumos' electrical contact thermometer (A. Gallenkamp and Co. Ltd.).

The period of incubation depended upon the activity of the preparation which was assayed previously.

e. Estimation of Enzyme Activity Enzyme activity was determined by estimation of the inorganic phosphate liberated from ATP during the reaction using the Atkinson, Gatenby and Lowe, (1973) modification of the method of Fiske and Subbarow (1925).

The reaction was stopped by the addition of 4ml of a freshly prepared mixture (1:1 (v/v) of 1% (w/v) aqueous 'Lubrol W' or 'Cirrasol ALN-WF', and 1% (w/v) ammonium molybdate in 1.8N H_2SO_4). A reagent blank was prepared by addition of the lubrol-molybdate medium before the enzyme was introduced. The tubes were left at room temperature (18-20°C) for 10 minutes to allow the yellow colour to develop, and then transferred to crushed ice for storage up to 1 hour. If necessary, precipitated protein was removed by centrifugation at 1000g for 15 minutes at 4°C in a 'Mistral 2L' Centrifuge (M.S.E. Ltd.).

The optical density of the supernatant was read against a distilled water blank at 390nm either in a Hilger and Watts

Ltd. Spectrophotometer or a Pye Unicam SP1800 Dual Beam Spectrophotometer, using a tungsten filament light source operating through a 10mm light path. Inorganic phosphate content was determined by reference to a calibration graph prepared by assay of standard phosphate solutions serially diluted from a stock solution containing 20ug phosphorus (as KH_2PO_4)/ml (figure 5-1). The response characteristics of assay mixtures prepared with Lubrol and Cirrasol were identical.

f. Protein Determination The protein content of microsomal preparations was estimated using the Folin-Ciocalteau method of Lowry, Rosebrough, Farr and Randall (1951), modified to permit the accurate determination of microsomal suspensions with low protein content (i.e. less than 100ug/ml.).

Reagents: Folin's Solution A: 50 vol, 3% (w/v) sodium carbonate in 0.15M NaOH.
0.5 vol, 0.75% (w/v) Copper sulphate.
0.5 vol, 1.5% (w/v) sodium potassium tartrate.
Folin's Solution B: 40% (v/v) aqueous solution of Folin's Ciocalteau-Phenol Reagent.

Procedure: Microsomal protein was diluted to give several known dilutions less than 100ug protein/ml. Standard protein solutions were made up of 0, 25, 50, 75 and 100ug Bovine Serum Albumin, Fraction V/ml. 1.2ml

of the standards, blanks and unknowns were added to 2.0 ml. of Folin's Solution A in a 150 X 25 mm boiling tube. After exactly 30 minutes 0.3 ml aliquots of Folin's Solution B was added to each tube. After a further 60 minutes the optical density of the resultant solution was measured against a distilled water blank, through a 10 mm light path in glass cuvettes at 650 mm. A calibration graph relating optical density to standard protein concentration was constructed (Figure 5-2) and the concentration of the unknowns determined from this line.

Enzyme activity was calculated as uMoles phosphate liberated / mg. protein / minute.

g. Calculation of the concentration of free calcium in a Calcium-Buffer System The presence of significant calcium contamination from extraneous sources and of an unknown number of Calcium-binding sites in the enzyme preparation, made it impossible to control the free calcium concentration accurately, particularly at the very low levels ($<10^{-5}M$) that are known to maximally activate the Ca^{2+}-Mg^{2+}-ATPase. This problem was overcome by the use of a Calcium-buffer system, using substances which selectively bind to divalent ions. One of the best known substances of this type is Ethylene-diamine-tetra-acetic acid (EDTA) which binds calcium about 10^2 times more effectively

than magnesium. Recently, however, EDTA has been superseded by Ethyleneglycol bis (ß-amino-ethyl-ether)-N, N-tetra-acetic acid (EGTA), since it can bind calcium 10^5 times more effectively than magnesium.

Normally only binding of divalent cation by the forms of EGTA with three negative charges (HL^{3-}) and four negative charges (L^{4-}) are considered (Caldwell, 1970). Thus at given conditions of pH, ionic strength and temperature, the apparent association constants K_1 and K_2 for the two forms of cation binding can be defined -

$$K_1 = \frac{(MeHL^-)}{(Me^{2+})\ (HL^{3-})}$$

$$K_2 = \frac{(MeL^{2-})}{(Me^{2+})\ (L^{4-})}$$

where (MeHL-) and (MeL^{2+}) represent the concentration of cation - EGTA complex for the two forms of binding, (HL^{3-}) and (L^{4-}) represents the total ligand that is not complexed, and (Me^{2+}) represents the uncomplexed cation concentration.

A combined apparent association constant K , may be defined, where -

$$K = K_1 + K_2 = \frac{(MeL)}{(Me^{2+})\ (L)}$$

(MeL) represents the total concentration of cation - EGTA complex

(L) represents uncomplexed ligand

(Me^{2+}) represents the remaining free cation concentration.

This latter equation was used to calculate the free calcium concentration using a value of K of $10^{6.882}$ (Caldwell 1970) by a process of successive approximation. Initially it was assumed that all available cation was bound by the ligand, so that (MeL) was equal to the total calcium present, and (L) was equal to the total ligand remaining uncomplexed. These values of K , (MeL) and (L) were used to calculate a value of (Ca^{2+}). Subsequently, the values of (MeL) and (L) were adjusted to account for the free (Ca^{2+}), calculated previously. This process was repeated until reasonably steady values of free (Ca^{2+}) were reached. The effect of magnesium on the equilibrium was not estimated.

The relationship between the total calcium in the system and the free calcium concentration in the presence of 0.5 mM EGTA is shown in Figure 5-3. All values of free (Ca^{2+}) were estimated from this graph.

Figure 5-1: Standard calibration curve for the determination of inorganic phosphate

Methods

Inorganic phosphate liberated during enzymatic activity was estimated using the method of Atkinson, Gatenby and Lowe (1973) with both 'Lubrol' and 'Cirrasol' reaction media (see 'Methods').

Legend

Ordinate - Absorbance at 390nm.

Abscissa - ug phosphorus (as KH_2PO_4) /ml.

o - 'Cirrasol' - (Hilger-Watts Spectrophotometer)

Δ - 'Lubrol' - (Hilger-Watts Spectrophotometer)

□ - 'Cirrasol' - (SP 1800)

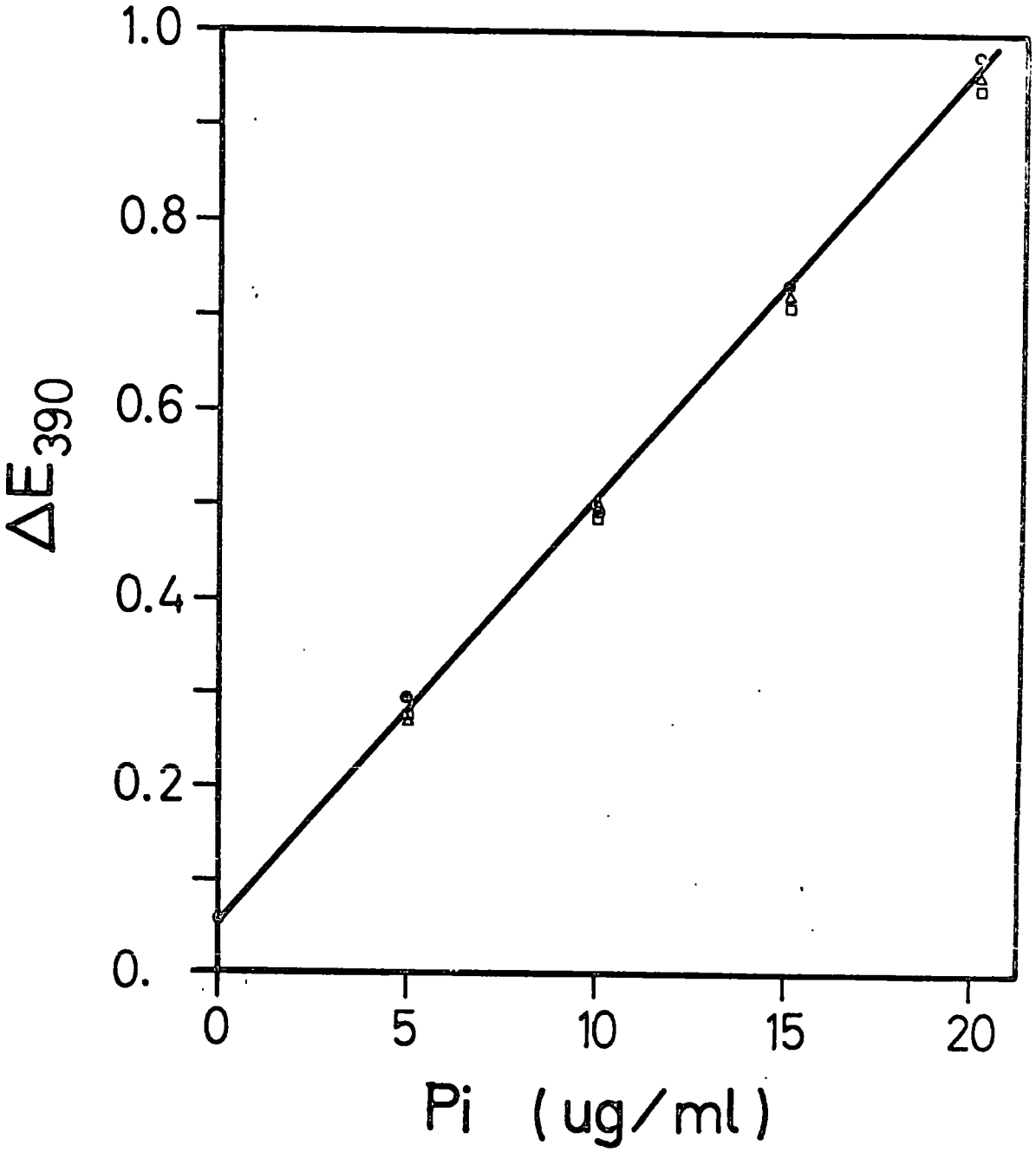

1.0
0.8
0.6
0.4
0.2
0.
ΔE390
0
5
10
15
20
Pi (ug/ml)

Figure 5-2: Standard calibration curve for the determination of Protein

Methods

Protein was estimated using the Folin-Ciocalteau phenol reagent, with BSA (fraction V) as standard. (See 'Methods'). Results are the mean of three separate determinations, each performed in duplicate.

Legend

Ordinate - Absorbance at 650nm

Abscissa - ug BSA (Fraction V)/ml

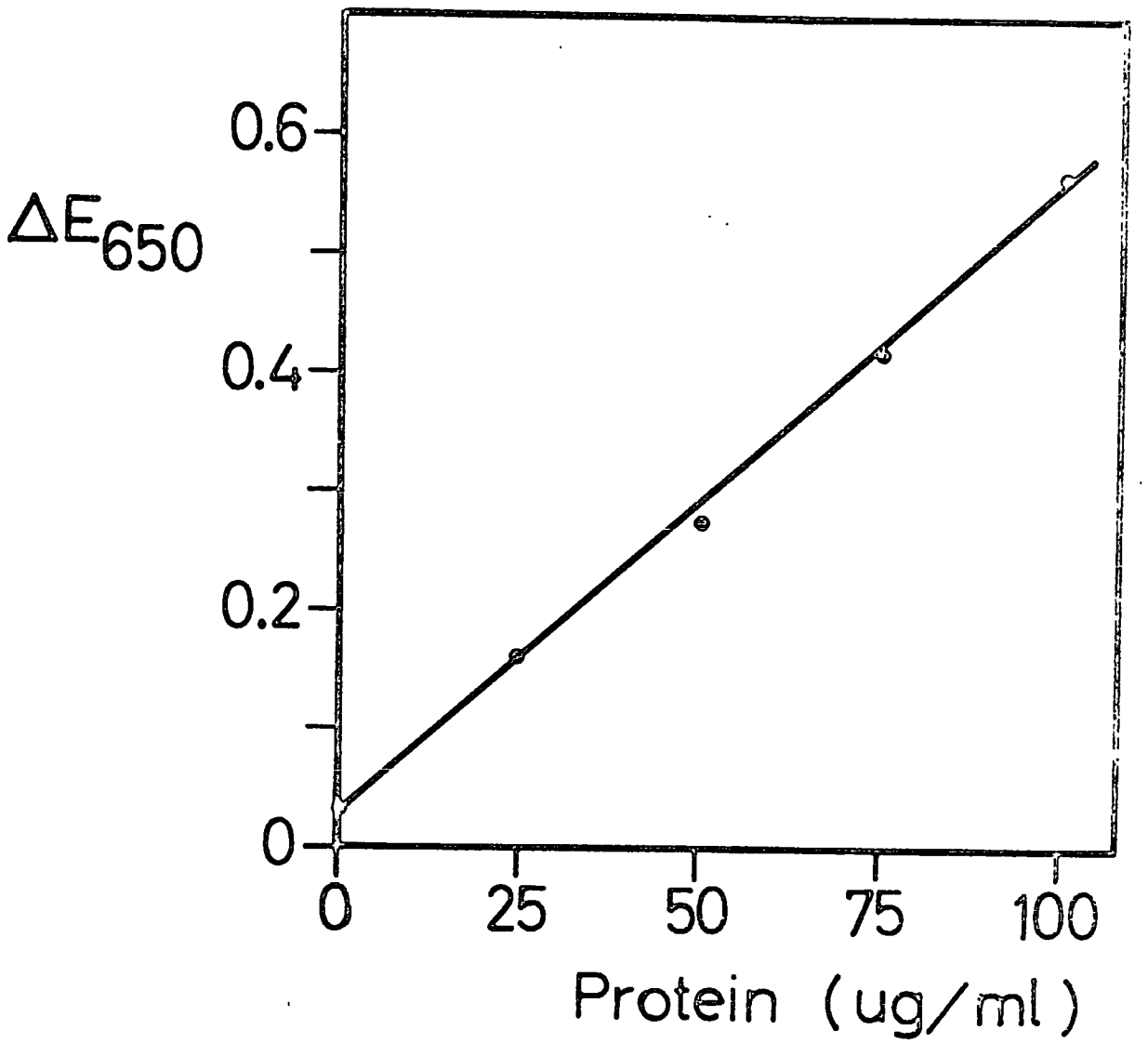
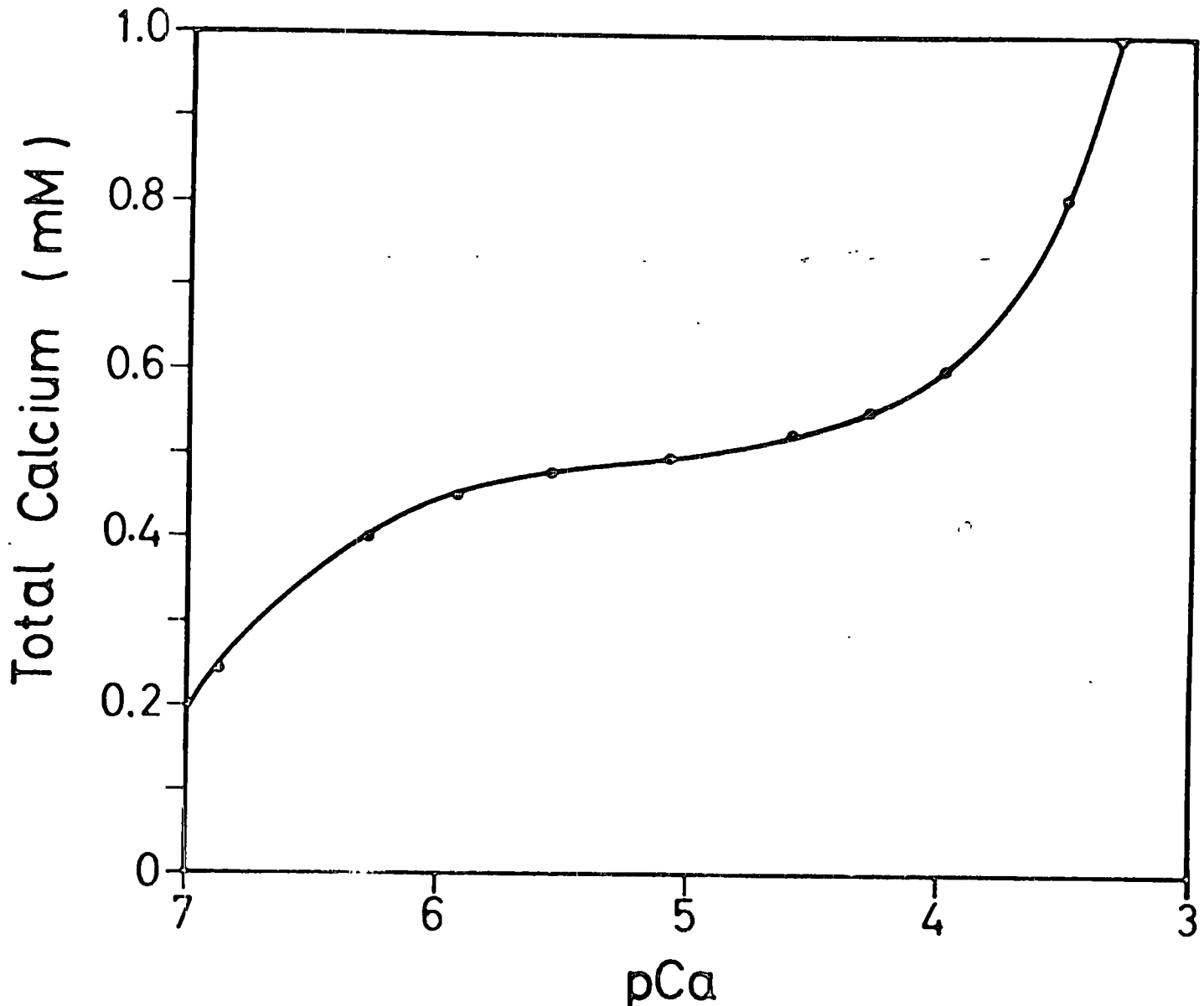

ΔE650
0.6
0.4
0.2
0
0
25
50
75
100
Protein (ug/ml)

Figure 5-3: A graph of the calculated free calcium concentration for a medium containing 0.5 mM EGTA and various concentrations of total calcium

Method: For calculations see 'Methods'. Dissociation constant for a mixture of Calcium and EGTA (at 20°C, pH 7.1 and in the presence of 0.1 M KCl) was $10^{6.882}$ (Caldwell, 1970).

Legend: Ordinate: Total Calcium concentration (mM)
Abscissa: pCa (free calcium concentration) (M)


1.0
0.8
0.6
0.4
0.2
0
Total Calcium (mM)
7
6
5
4
3
pCa

Chapter 6

THE EFFECT OF TEMPERATURE UPON THE KINETIC CHARACTERISTICS OF THE SARCOPLASMIC $Ca^{2+}Mg^{2+}$ATPase OF CRAYFISH ABDOMINAL MUSCLE

INTRODUCTION

The Immediate Effects of Temperature upon Enzyme Kinetics

It has been known for some time that an increase in temperature causes a marked stimulation in the rate of a chemical reaction. In the 1880's Arrhenius offered an explanation of this by suggesting that for a chemical reaction to proceed, the reactants must possess a particular amount of energy which he termed the 'Activation Energy'. This threshold value could be calculated from the empirical Arrhenius Equation (which relates Activation Energy to the temperature dependence of the reaction velocity), and was thought to be characteristic for any particular reaction sequence.

However, amongst the most interesting observations of recent years has been that the Arrhenius Activation Energy of some enzymic reactions was markedly dependent upon substrate concentrations. Thus at low substrate concentrations, the reaction velocity was markedly less temperature-dependent. (Hochachka,1971; Hazel,1972a) This phenomenon was explained by the dependence of the Enzyme-Substrate (E-S) affinity (as measured by the Michaelis constant, Km) on the assay temperature. (Helmreich and Cori,1964; Taketa and Pogell,1965; Himes and Wilder,1968.) In some cases the dependence had a linear relationship (succinic dehydrogenase, Hazel 1972; pig isocitrate dehydrogenase, Moon 1972; Adenosine transport, Berlin 1973), whilst in others it proved

to be curvilinear (lactate dehydrogenase, Hochachka and Somero 1968, Hochachka and Lewis,1971, Baldwin and Aleksuik, 1973; goldfish brain choline acetyl-transferase, Hebb, Stephens and Smith,1972; isocitrate dehydrogenase, Moon and Hochachka,1971; glucose-6-phosphate dehydrogenase, Somero,1969; fructose diphosphatase, Behrisch and Hochachka, 1969; acetylcholinesterase, Baldwin and Hochachka,1970), or a complex U-shape (lactate dehydrogenase, Somero,1969; pyruvate kinase, Somero and Hochachka,1968; see Figure 6-1). Berlin (1973) has observed a linear relationship between Km and temperature in the trans-membrane transport of adenosine in macrophages. In all of these cases an increase in assay temperature above the adaptation temperature of the organism caused a reduction in the E-S affinity (increase in Km). This resulted in a reduction of the reaction velocity which counteracted partially or totally the rate-stimulating effects of increased temperature. The physiological significance of this phenomenon is that it provides a potential mechanism for escaping the possible deleterious effects of increased temperature upon the delicately balanced state of cellular metabolism. It has been termed "positive thermal modulation" by Hochachka and his colleagues (Hochachka and Somero 1973).

The degree of thermal compensation achieved depends upon the extent of the change in Km with temperature, and also upon the substrate concentration. Thus a steep Km/temperature curve would result in a greater degree of thermal independence than a shallow Km/temperature curve. It is interesting to note that eurythermal organisms, such as Gillichthys sp. (Somero 1973) which may experience diurnally fluctuating temperatures, tend to possess enzymes

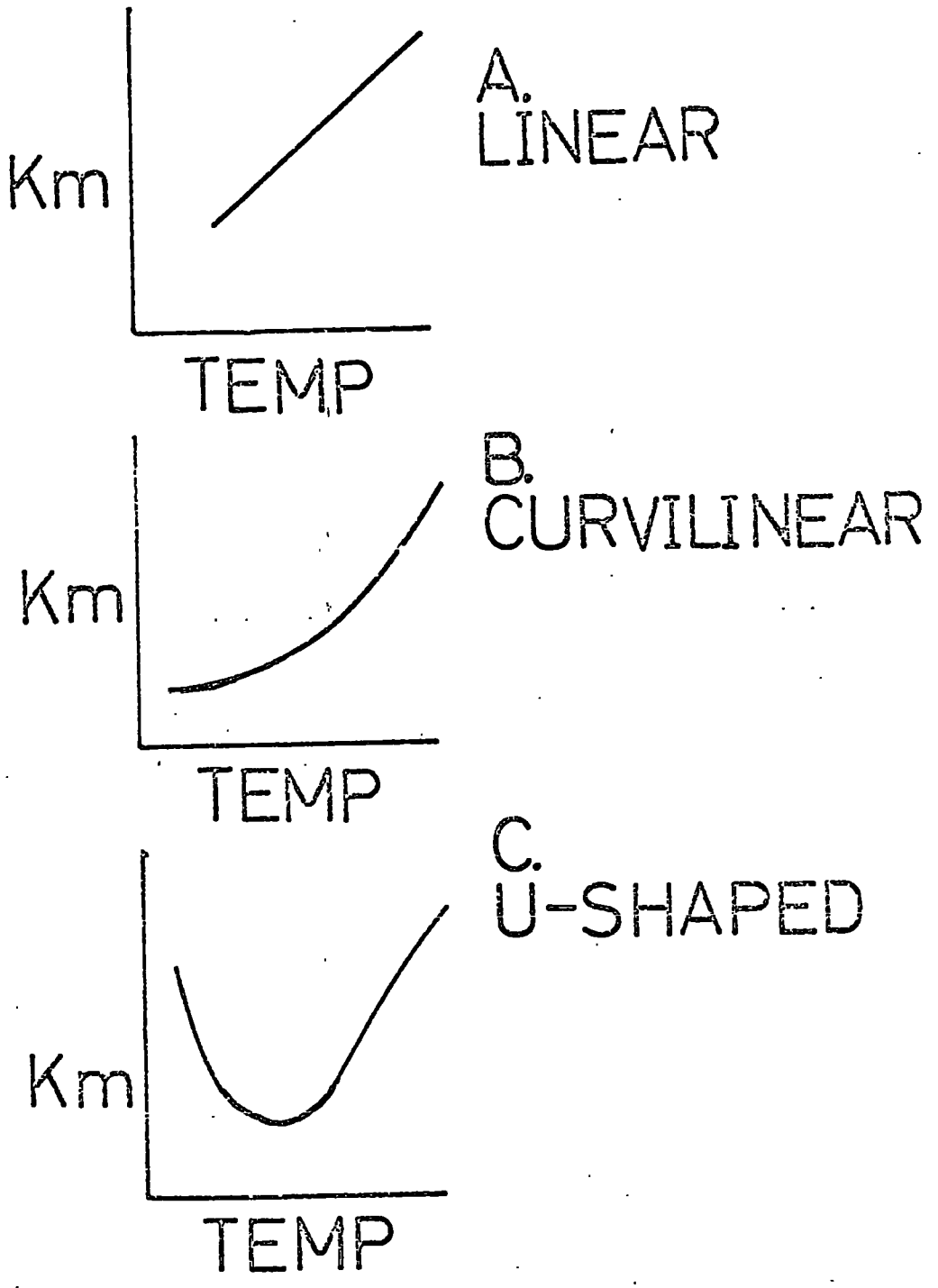


Figure 6-1. Types of Km / Temperature Plot

(See Text)

with shallow Km/temperature curves which permit efficient enzyme function over a wide range of temperatures. Stenothermal organisms, however, often have steep Km/temperature curves which result in a degree of temperature independence, but restricted limits for enzymic function (Trematomus sp. Hochachka and Somero 1973). These phenomena may well have ecological implications. At low substrate concentrations, which are presumed to be present intracellularly (Williamson, Cheung, Coles and Herczeg,1967; Hochachka, Freed, Somero and Prosser,1971; Freed,1971a), the E-S affinity is of primary importance in governing the rates of catalysis. At saturating levels of substrate, however, the intrinsic enthalpy of the system is the major determinant of reaction velocity. For example, King Crab (Paralithodes sp.) lactate dehydrogenase at a pyruvate concentration of 2mM has a Q_{10} (5-15°C) of 1.8, whereas at 0.1mM pyruvate it is 1.2 (Somero 1969). At 0.05mM pyruvate, there is overcompensation with a Q_{10} of 0.8.

In addition to the immediate compensating effects discussed above, complex U-shaped curves relating Km to temperature display several other important characteristics. Firstly, some poikilothermic enzymes exhibit maximal E-S affinity (minimal Km value) at temperatures which approximate to the habitat (and body) temperature (Somero and Hochachka,1969; Somero,1969; Baldwin,1971). This is a classical adaptive relationship and supports the contention that it is physiologically significant and the result of evolutionary processes. Secondly, the marked increase in Km at temperatures below those normally experienced by the animal may be expected to act additively with the reduction in kinetic energy, to stop effectively the reaction proceeding.

On first considerations this situation appears paradoxical, but Somero and Hochachka (1973) have speculated that dramatic increases in Km, within the viable thermal range of an organism, may be instrumental in switching metabolic activity from one pathway to another. They feel that the resulting metabolic reorganisation may be adaptive. Furthermore, at extreme temperatures cessation of enzyme activity may cause complete inactivation of a metabolic pathway, which, even though the enzyme is not irreversibly inactivated, may prove lethal to the organism concerned. Thus heat death, and more likely cold death, may follow in the absence of protein denaturation.

These proposed mechanisms by which enzymes counteract the rate-effects of increased temperature have certain implications. Firstly, as they are essentially immediate, no synthesis of protein is required. Secondly, such compensation would be automatic and requires no special controlling machinery. Thirdly, it provides an explanation at the molecular level for recent claims of thermal independence of certain physiological processes (Newell and Northcroft,1967; Newell and Pye,1971; Percy and Aldrich,1971). In addition, the magnitude of the temperature-dependent change in Km is often dependent on a variety of factors other than temperature. These include enzyme concentration (Hochachka and Lewis,1971), the pH of the medium (Hochachka and Lewis,1971; Helmreich and Cori,1964; Himes and Wilder, 1968; Freed,1971a), the presence of mono- and divalent cations (Taketa and Pogell,1965; Behrisch and Hochachka, 1969) and the presence of other metabolites (Lowry, Schultz and Passonneau,1964).

Indeed, so rudimentary is our understanding of the intracellular environment, that we do not know the substrate concentration experienced by an enzyme. The intimate compartmentalisation that exists within cells ensures that a measurable bulk concentration is not evenly distributed (Bygrave, 1967). Neither are we sure that the enzyme kinetics observed *in vitro* bear any relation to *in vivo* conditions, since conventional assay systems differ markedly from their predicted kinetic and regulatory behaviour at the high enzyme concentrations that exist within the cell (Srere, 1967; Wuntch, Chen and Vesell, 1970; Horvath and Sovak, 1973). Weitzman (1973) has suggested that the use of "permeabilised" cells may be a simple technique of simulating *in vivo* conditions, since treatment with toluene leads to total incontinence towards low molecular weight molecules, whilst the macromolecular components are retained intracellularly. Not only are the enzymes present at intracellular concentrations but there is preservation of at least some higher-order macromolecular interactions, characteristic of living cells.

The molecular mechanism of temperature modulation of E-S and enzyme modulator affinity is largely unknown. Lowry, Schulz and Passonneau (1964) have suggested that increased temperature causes a conformational change which offers poor accessibility of sites to substrates and allosteric modulators, leading to increased Michaelis constants. The tertiary and quaternary structures of enzymes are known to be determined primarily by weak hydrophobic, dipole and ionic interactions between various parts of the polypeptide chain. Increases in temperature cause marked diminution of the strenth of ionic interactions and hydrogen bonding by

an increase in thermal mobility of the molecule. In addition, temperature changes may have multiple effects on the state of ionisable groups, particularly those with appreciable heats of ionisation. Similarly, temperature decreases would be expected to cause an increased lability of hydrophobic interactions, a factor which is of obvious importance to intrinsic membrane-bound enzymes. Hochachka and Somero (1973) have speculated as to the modifications of the primary protein structure which would engender stability to the overall enzyme structure or its active site at particular temperatures, and Smith (1970) has listed the roles that the side chains of each of the twenty common amino-acids are known or presumed to play in the structural and functional properties of enzymes. Such interactions between temperature and protein conformation are no doubt extremely complex in nature, but there is good experimental evidence for their existence (Iwatzuki and Okazaki, 1967).

The Role of Isoenzymes in Temperature Acclimation

On a seasonal time scale, effective compensation for an altered thermal regime may be promoted by the production of kinetically distinct isoenzymes, each operating efficiently over different temperature ranges. Often those enzymes with season-specific isoenzymes, are those which exhibit the more dramatic increases of Km at temperatures much removed from their adapted thermal regime, and thus are functional over a limited temperature range only. Accordingly, isoenzymes of pyruvate kinase, which differ kinetically, have been demonstrated in extracts isolated from Rainbow Trout, acclimated to 2°C and 18°C (Somero and Hochachka, 1968, 1971). Other examples are the choline acetyltransferase of the Goldfish (Hebb, Stephens and Smith, 1972), the acetylcholinesterase of

Trematomus sp. and Rainbow Trout (Baldwin and Hochachka, 1970), the NAD-dependent isocitrate dehydrogenase from trout liver (Moon, 1970; Moon and Hochachka, 1972); trout liver citrate synthetase (Hochachka and Lewis, 1970); trout phosphofructokinase (Somero and Hochachka, 1971) and the oxoglutarate aminotransferase of the Pond Loach (Mester, Iordăchescu and Niculescu, 1973). In all of these examples each isoenzyme exhibited maximal E-S affinity at temperatures which approximated to the acclimation temperature. Furthermore, Livingstone and Bayne (1974) have noted marked seasonal changes in the E-S affinity of the mantle pyruvate kinase of a natural population of the edible mussel, Mytilus edulis, that are consistent with the known seasonal glycolytic and gluconeogenic properties of this tissue. This is thought to indicate a correlation between isoenzyme production and a change in metabolic function of the tissue.

The production of functional enzyme variants is known to occur by several means, although the data are not extensive enough to permit generalisation of the importance of each mechanism to any one tissue, organ or animal. In this context it is relevant to note that the shorter the time-course in which thermal compensation occurs, the more limited are the enzymatic modifications that may constitute an animal's adaptive response. Immediate thermal dampening effects as described previously, can only be completed with the existing stock of enzymes. However, short-term or seasonal acclimation requires the production of different forms of the same enzyme, each with different temperature characteristics. This may be effected by the clear-cut, on-off synthesis of isoenzymes as has been demonstrated with Trout brain acetylcholinesterase (Baldwin and Hochachka, 1970) and the intestinal alkaline

phosphatase of Salvelinus sp. (Whitmore and Goldberg,1972). With oligomeric enzymes, kinetic variants may be produced in certain tissues by changing the balance of subunit types (Hochachka,1965, 1966; Somero and Hochachka,1969; Hochachka and Lewis,1970, 1971; Aleksiuk,1971; Moon and Hochachka,1971; Whitmore and Goldberg,1972; Somero,1973; Bolaffi and Brooke, 1974). However, Wilson, Whitt and Prosser (1973) have stressed caution in evaluating electrophoretic data on isoenzyme composition since they observed significant differences in lactate dehydrogenase isoenzyme patterns of Goldfish muscle, that were due to causes other than temperature acclimation. These include enzymic polymorphisms in the ratio of A and B subunits; variations in the ratio of red and white muscle fibre types, each with distinct subunit compositions; and inconsistencies in staining technique (see also Bolaffi and Brooke,1974).

It is implicit in this description that isoenzyme and subunit replacement techniques require a sophisticated controlling mechanism to produce the right molecules at the right time.

Modulation of Enzyme Activity during Acclimation

Functionally distinct isoenzymes may also be produced by modulation of the kinetic and regulatory behaviour of an existing protein, resulting in an enzyme which is more efficient and relevant to the new environmental conditions. The effects of the intracellular environment upon enzyme function is of particular relevance to metabolic control in poikilotherms during temperature acclimation, since pH (Rahn,1965; Reeves and Wilson,1969; Freed 1971a) and mono- and divalent cation content (Rao,1962; Hickman, McNabb, Nelson, Van Breenan and

Comfort,1964; Heninicke and Houston,1965; Houston and Madden, 1968) are known to be regulated during seasonal thermal acclimation in fish. Hochachka and Somero (1971) have concluded, from a series of detailed studies on the thermal characteristics of certain important regulatory enzymes, that although individual enzyme-modulator interactions may be temperature sensitive, as described previously, the overall regulation of catabolic function may be highly independent of temperature (Somero and Hochachka,1968; Behrisch,1969). This suggests that it is the adapted intracellular environment that causes modifications to the kinetic and/or regulatory properties of the enzyme, presumably by allosteric interaction. In support of this hypothesis, Freed (1971a) has suggested that the decreased glycolytic rates and increased levels of fructose-6-phosphate and glucose-6-phosphate, observed in warm acclimated goldfish may be explained by the demonstrated modulating effects of decreased pH on phosphofructokinase activity. Clearly such modifications are time-dependent and are quite distinct from the immediate effects of temperature upon the enzyme-substrate and enzyme-modulator affinity discussed earlier.

Almost all the work discussed previously, apart from the exhaustive analysis of goldfish succinic dehydrogenase by Hazel (1972), has been performed on "soluble" enzyme systems assayed *in vitro*. The limitations of this approach have been discussed. The fragmented sarcoplasmic reticulum (FSR) preparations used in the present study offers some distinct advantages over "soluble" enzyme preparations, since the enzymes are in a localised concentration and three-dimensional arrangement that resembles the *in vivo* situation more closely. Consequently, the interactions between various

macromolecules are largely maintained, although no doubt in a modified form. Furthermore, the vesicles retain the correct orientation for transport.

The experiments described in this chapter were designed to investigate the temperature-dependent kinetic characteristics of the Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase of the FSR. In particular, tne Km/temperature relationship was determined and the possible involvement of Km changes in temperature-independent functioning, investigated. Also, the functional characteristics of enzymes isolated from $4^{o}C$ and $25^{o}C$ acclimated crayfish were compared to see if the phospholipid environment which is affected by thermal acclimation (see Chapter 3) caused any compensatory changes in enzyme behaviour.

MATERIALS AND METHODS

A. MATERIALS

a. Animals. Adult crayfish between 30mm and 50mm carapace length were acclimated either to $4^{o}C$ or $25^{o}C$ for at least 21 days as described in Chapter 2. All animals were subjected to a 18-hour daylength artificial light conditions. Particular care was taken to ensure that animals were in the intermoult condition when sacrificed.

b. Skeletal muscle. The abdominal flexor and extensor muscles from each animal were used. Usually muscle tissue from up to ten animals was pooled to provide sufficient material for analysis.

c. Reagents. The composition of all extraction, resuspension and assay media has been described in Chapter 5. Variations to the concentration of Tris-ATP, EGTA and calcium are noted in the text at relevant points.

B. METHODS

a. Preparation of the microsomal fraction. The preparative procedure has been described in Chapter 5. The microsomal suspension was either used immediately or stored for up to 1 hr on ice. For long term storage (1 hr to 7 days) aliquots were stored separately at -20^{0}C in 0.3M sucrose, 10mM imidazole at pH 7.1. These aliquots were thawed approximately ten minutes prior to assay and stored on ice as above.

b. Enzyme assays. Basal Mg^{2+}-ATPase, Ca^{2+}-dependent ATPase and total Ca^{2+}-Mg^{2+}-ATPase activities were usually assayed in duplicate as described in Chapter 5. The reaction was stopped and the inorganic phosphate liberated was determined as described previously. Protein determinations were performed in triplicate using the method of Lowry, Rosebrough, Farr and Randall (1951) as described in Chapter 5.

c. Statistical techniques

(i) Statistical comparisons of data. All statistical comparisons of data were performed with conventional techniques as described by Snedecor and Cochran (1967). Where appropriate, reference was made to the statistical tables of Fisher and Yates (1963).

(ii) Treatment of saturation kinetics data. The Michaelis-Menten equations as modified by Lineweaver and Burk (1934), permits the graphical estimation of the kinetic parameters Km and Vmax (Dixon and Webb, 1964). However, since there was a degree of scatter in the correlation between $^{1}/_{V}$ and $^{1}/_{S}$, the kinetic constants were determined by the method of least squares (Snedecor and Cochran, 1967).

$$\text{Regression Coefficient} = b = \frac{\Sigma XY - \frac{\Sigma X \Sigma Y}{N}}{\Sigma X^2 - \frac{(\Sigma X)^2}{N}} \quad \text{Equation - 6-1}$$

where the symbols ΣX, ΣY, ΣXY and ΣX^2 have their usual meanings, and N - number of observations.

$$\text{The intercept } a = \frac{1}{V} = \frac{\Sigma Y}{N} - b.\frac{\Sigma X}{N} \quad \text{Equation - 6-2}$$

As a check on equation 6-2, the intercepts of the extrapolated line on the $^{1}/_{S}$ axis were calculated from:

$$X = \frac{-1}{Km} = \bar{X} - \frac{(Y-\bar{Y})}{b}$$

Since in this instance Y = 0, this reduces to

$$X = \bar{X} - \frac{\bar{Y}}{b}$$

$$\text{and } Km = \frac{1}{\left(\bar{X} - \frac{\bar{Y}}{b}\right)}$$

The regression analysis provides a "best estimate" fit of the regression line for the available data. However, it is necessary to recognise that each velocity determination has a certain error associated with it, and that this results in an error in the regression coefficient -b, and in any

prediction made from the regression data.

The standard error of the regression coefficient (Sb) can be calculated from:

$$S_b = \sqrt{\frac{\frac{\Sigma y^2}{\Sigma x^2} - b^2}{N - 2}} \qquad \text{Equation 6-3}$$

where $\Sigma y^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{N}$ and $\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{N}$

The value and standard error of a value of X predicted from this regression (Snedecor and Cochran 1967) is given by:

$$X = \overline{X} + \left(\frac{Y - \overline{Y}}{b}\right) \pm Sb \sqrt{\Sigma x^2 + \frac{\Sigma x^2}{N} + \frac{(Y - \overline{Y})^2}{b}}$$

Since at the intercept on the X axis Y = 0, and $-X = -\frac{1}{Km}$ this becomes:

$$X = \left(\overline{X} - \frac{\overline{Y}}{b}\right) \pm \frac{Sb}{b} \sqrt{\Sigma x^2 + \frac{\Sigma x^2}{N} + \frac{\overline{Y}^2}{b^2}} \qquad \text{Equation 6-4}$$

Confidence limits for X were estimated in the usual manner from the Students 't' distribution by:

$X \pm$ t. (Standard error of X).

where t is the desired level of significance for (N-2) degrees of freedom (Fisher and Yates, 1963). Km, its standard error and confidence limits, were found by taking reciprocals of these values. It should be noted, however, that by taking reciprocals of (X + t x S.E) and (X - t x S.E) the resulting confidence intervals above and below Km, will not be equal (see Figures 6-14 and 6-15).

The double reciprocal plot is the most often used of the several straight line

conversions available (Dixon and Webb 1964), but it may be the least reliable source of estimates of Vmax and Km. Several linear methods have been compared by Dowd and Riggs (1965) who tested them using simulated data and investigated the sensitivity of each method to error in the velocity measurement. They found that the double reciprocal plot gave a deceptively good fit, even with unreliable experimental values. If used without adequate weighting it can result in large errors in the kinetic constants since the smallest value of v plays an inordinately important role in determining the position of the fitted line. Of the three methods examined it proved to be the least reliable.

When the error in v is large Dowd and Riggs (1965) concluded that more consistent estimates of Vmax and Km are given by plotting v against $v/[S]$. It has the further advantage of indicating when the data deviates from the predicted relationship, since it tends to exaggerate such deviations.

The basic Michaelis-Menten equation is multiplied through by (S + Km) and rearranged to give:

$$v = Vmax - \frac{v\,Km}{[S]} \qquad \text{Equation } 6\text{-}4$$

which is of the general form $y = m - \frac{y}{x}\,C$. (Dixon and Webb, 1964). A plot of y against y/x gives a line whose intercept on the ordinate is m (= Vmax) and whose slope is c (= Km).

These parameters and their associated standard errors were calculated by regression analysis as indicated previously for the Lineweaver-Burk plot. In this case the confidence intervals above and below Km will be equal (see Figures 6-14 and 6-15).

RESULTS

A. Reaction Characteristics of the Microsomal ATPase

Extraction Characteristics

The initial pellets A and B (see General Methods, Chapter 5) were usually white and were thought to consist largely of myofibrillar material, muscle nuclei, and connective tissue. Pellet C was white or slightly pink in colour and consisted of remaining myofibrillar material, nuclei and mitochondria. The heavy microsomal pellets D and E were both straw-coloured or pink, and lent an opalescent quality to the medium when resuspended.

The extraction efficiency is a measure of the yield of microsomal protein from each gram wet weight of muscle. It was calculated that the yield of microsomal protein from crayfish muscle was between 0.6 - 0.9mg microsomal protein per gram wet weight of muscle. On average the abdominal muscle from 25°C-acclimated crayfish yielded more microsomal protein than 4°C-acclimated crayfish abdominal muscle. This difference is highly significant ($P < 0.01$, see Table 6-1).

Characterisation of ATPase Activity of the Heavy Microsomal Fraction

The following experiments were designed to characterise the ion requirements for ATPase activity of the heavy microsomal preparation from crayfish abdominal muscle,

and to investigate the presence of other Ca^{2+}-activated ATPase systems that may contribute to the observed Ca^{2+}-stimulation of ATP hydrolysis.

(i) Basal Mg^{2+}-dependent ATPase: The microsomal preparation possessed an appreciable ATPase activity when assayed in the presence of 3mM $MgCl_2$, but absence of $CaCl_2$ and EGTA. Incorporation of increasing concentrations of EGTA into the reaction medium caused a reduction in the rate of ATP hydrolysis (Figure 6-2). ATPase activity was almost totally suppressed in the presence of 0.5mM EGTA.

In most preparations (60% or more), Mg^{2+}-dependent ATPase activity in the present of 0.5mM EGTA was toally absent, but when present was of very low activity, never exceeding 8% of the 'Total' Ca^{2+}-Mg^{2+}-ATPase activity in preparations isolated from both $4^{o}C$ and $25^{o}C$-acclimated crayfish (Table 6-2). Whether this activity was a component of the FSR or was a contaminant from other membrane systems is not known.

(ii) ATPase Activity in the absence of Mg^{2+}: No ATPase activity was observed at a free Ca^{2+} concentration of 8.1uM, in the absence of Mg^{2+}. This indicates that no Ca^{2+}-dependent ATPase activity exists under the reaction conditions routinely used (Table 6-2).

(iii) Effect of Sodium Azide: The incorporation of 5mM sodium azide into a reaction medium containing 8.1uM free calcium and 3mM $MgCl_2$ caused no reduction in the rate of ATP hydrolysis, when compared to a control reaction without sodium azide. This indicates that a mitochondrial, Ca^{2+}-stimulated ATPase does not contribute to the observed ATP hydrolysis since

Fanberg and Gergely (1965) have shown that both ATPase activity and calcium uptake by a mitochondrial fraction were inhibited by sodium azide.

(iv) Ca^{2+}-activation of the Mg^{2+}-dependent ATPase:

Incorporation of low concentrations of free calcium (pCa <6) into a reaction medium containing 2mM $MgCl_2$, caused a marked stimulation of ATP hydrolysis (Figure 6-3). However, incorporation of larger quantities of free calcium (pCa >5) resulted in the inhibition of the Ca^{2+}-stimulated rate of ATP hydrolysis, revealing a clear optimum at 20°C in the presence of 7uM free calcium.

B. The Effect of Assay Temperature and Thermal Acclimation upon the Ca^{2+}-activation Characteristics of the Mg^{2+}-dependent ATPase

Time-course of ATP Hydrolysis with 3.0mM ATP

Figure 6-4 is a plot ATP hydrolysis with respect to time by the Ca^{2+}-stimulated ATPase, at an ATP concentration of 3.0mM and in the presence of 3mM $MgCl_2$, 0.5mM $CaCl_2$ and 0.5mM EGTA. The rate of ATP hydrolysis remained linear with time for the entire course of the experiment. On the basis of this result, incubation periods of 60, 30 and 10 minutes were adopted for assay at 5°C, 20°C and 35°C respectively.

Temperature and Acclimation Effects upon Ca^{2+}-activation

Measurements of the complex activation-inhibition characteristics of Ca^{2+}-stimulated ATPase activity induced by different concentrations of free calcium were made at 5°C, 20°C and 35°C for preparations extracted from 4°C-acclimated (Figure 6-5) and 25°C-acclimated crayfish (Figure 6-6).

The free calcium concentration required for maximal activation of ATPase activity varied between 2uM and 14uM. These small changes in optimal free calcium concentration at different assay temperatures were negatively related to temperature in a linear fashion (Figure 6-7a) and are probably caused by small temperature-induced shifts in the pH of the assay medium (see Figure 6-7b). However, the variations in the optimal free concentration were small in extent and in all subsequent analyses the free calcium concentration was maintained at 8.1uM using an equimolar solution of EGTA and calcium chloride. The calculated error in enzyme activity[3], caused by estimating optimal enzyme activity with 8.1uM free calcium and not the true optimal concentration for each temperature is indicated in Table 6-3 for microsomal preparations from both 4°C-acclimated and 25°C-acclimated crayfish. All errors are within 3% of the true optimal Ca^{2+}-stimulated activity, and lie within the estimated experimental error for the analytical technique ($\pm$5%).

The free calcium concentration required for maximal activation of Ca^{2+}-stimulated activity was identical at each assay temperature for preparations isolated from 4°C and 25°C-acclimated crayfish (Table 6-3 and Figures 6-5 and 6-6). It was therefore concluded that thermal acclimation had no effect upon the Ca^{2+}-activation characteristics of the enzyme.

The results also indicate that the Ca^{2+}-stimulated ATPase activity was markedly temperature dependent (see Chapter 7).

C. ATP Saturation Characteristics

Time-course of ATP Hydrolysis at 0.1mM ATP

Figure 6-8 shows a plot of the time course of phosphate liberation by the ATPase preparation at 0°C and 35°C, in the presence of 0.1mM ATP.

The Ca^{2+}-stimulated ATP hydrolysis remained linear for 10 minutes at 35°C and at least 50 minutes at 0°C. In the presence of Mg^{2+} only, no phosphate was liberated. On the basis of this result incubation times were adopted during which similar amounts of phosphate were liberated. The incubation time varied with the activity of each preparation.

Saturation of the Ca^{2+}-stimulated ATPase with ATP

Figure 6-9a shows the saturation curves with respect to ATP for a microsomal Ca^{2+}-stimulated ATPase preparation isolated from 4°C-acclimated crayfish. Careful examination of the linear plot indicates that the curve does not conform to the simple rectangular hyperbola predicted by Michaelis-Menten kinetics, but shows stimulated activity at ATP concentrations in excess of 0.5mM. This phenomenon is more clearly illustrated in the accompanying Lineweaver-Burk plot (Figure 6-9b), which reveals a complex graph consisting of two straight lines intersecting at approximately 0.5mM ATP.

For analytical purposes the two lines were taken to represent separate enzyme systems. Extrapolation of each line towards the ordinate permitted the separate determination of Vmax and Km for each system. Thus, below 0.5mM ATP (hereafter termed 'low [ATP] activity') the enzyme has an apparent Km of 72uM and a Vmax of 0.115uM phosphate liberated. mg protein^{-1}. minute^{-1}..

Above 0.5mM ATP (i.e., 'high [ATP] activity'), however, Km was increased dramatically to 285uM and Vmax to 0.156uM phosphate liberated.mg protein^{-1}. minute^{-1}, an increase of 35.6% over the Vmax for low [ATP] activity.

0.1mM proved to be the lowest practical substrate concentration at which reaction velocity could be estimated accurately. At lower ATP concentrations the reaction was not linear for long enough to permit accurate phosphate assay with the 'Lubrol' technique. Indeed, errors of ATPase activity measurements are generally magnified since each activity estimate requires the simultaneous phosphate determination in the presence and absence of calcium. Errors in either determination may affect the final result.

The Effect of Different Assay Temperatures on the Enzyme-Substrate Affinity

A single preparation from each acclimation group was examined. The ATP saturation curves at eight assay temperatures between 0°C and 35°C for preparations from both 4°C and 25°C-acclimated crayfish are presented in Figures 6-10 and 6-11, with their corresponding Lineweaver-Burk plots in Figures 6-12 and 6-13 respectively. (The basic data is presented in Tables 6-4 and 6-5.) It is clear that enzyme activity is very temperature dependent and that the complex double-reciprocal plot noted previously was evident at each assay temperature. The effect of temperature upon the enzyme activity of each preparation is discussed in Chapter 7.

The kinetic Constants Vmax and Km were calculated for both high and low [ATP] activities at each temperature, using two linear methods (Dixon and Webb 1964):

1. Lineweaver-Burk plot of $\frac{1}{v}$ against $1/[ATP]$
2. Eadie-Hofstee plot of v against $v/[ATP]$

In both cases the data was arranged as above and the "best fit" line estimated by regression analysis. The Michaelis constant Km, its standard error and Vmax were determined from the "best fit" line (see 'Methods') The regression data and the predicted values of Km and Vmax at high and low [ATP] activities are presented in Tables 6-6 and 6-7 for the 4°C-acclimated preparation and in Tables 6-8 and 6-9 for the 25°C-acclimated preparation.

The data derived from the Lineweaver-Burk transformation is in good agreement with that derived from the Eadie-Hofstee plot (see Tables 6-6 to 6-9), although the standard error of the Km estimates from the latter plots were generally smaller. The effects of temperature upon Km (ATP) are illustrated graphically in Figures 6-14 and 6-15.

(i) Activity at low ATP concentrations. The 4°C-acclimated preparation had a Km (ATP) which varied slightly between 70uM and 90uM over the temperature range 0°C to 28°C, with a mean of 80.47uM (Table 6-7 and Figure 6-14a). The 25°C-acclimated preparation also showed variation between restricted limits (one point excepted, Figure 6-14b) over the same temperature range but with reduced mean Km of 70.57uM (Table 6-9 and Figure 6-14b). The difference in mean Km between 4°C and 25°C-acclimated preparations was not statistically significant (Table 6-10; P 0.2). In both preparations the observed variations in Km between 0°C and 28°C were somewhat fickle in magnitude by comparison with the expected error in each Km determination, and inconsistent in direction (Figure 6-14). It was therefore concluded that they probably represent

different estimates of the same true value of Km, and that Km was independent of temperature over the range 0°C to 28°C.

Assay at 35°C, on both preparations caused a significant increase in Km to 148uM in the 4°C-acclimated preparation, and 111uM in the 25°C- acclimate preparation. Both values are significantly higher than the mean Km between 0°C and 25°C (Table 6-11).

(ii) Activity at high ATP concentrations. The variations of Km with temperature at higher concentrations of ATP (Tables 6-6 and 6-8) are illustrated graphically in Figure 6-15a for the 4°C-acclimated preparation and Figure 6-15b for the 25°C-acclimated preparation. Once again there was some variation in the value of Km at different assay temperatures, though it was more marked in the 25°C-acclimated preparation. Similarly, the standard error of Km was greater and the correlation coefficient less significant in the 25°C-acclimated preparation (Table 6-6) than in the 4°C-acclimated preparation. No doubt this is largely explained by the smaller number of points in the former analysis. The difference in the mean Km between the preparations isolated from 4°C and 25°C-acclimated crayfish was not significant ($P < 0.70$, Table 6-10).

In the 4°C-acclimated preparation Km showed a small decrease with increasing temperature, but rises at 23°C followed by another decrease above this temperature (Figure 6-15a). These variations, however, are small and each value lies fairly closely to the mean Km. The overall trend is most probably

that Km does not vary with temperature.

On the first appearances the 25°C-acclimated preparations have a somewhat different trend line (Figure 6-15b). The high value of Km (ATP) at 0°C declines as the assay temperature is increased from 0°C to 25°C with a further slight increase above this temperature. The interpretation relies heavily on the value of Km at 0°C, which although it has a high correlation coefficient and low standard error, appears at 670uM to be excessively high. If this value is ignored the variation in the trend line becomes small and one can only conclude, for similar reasons as for the preparation from 4°C-acclimated animals, that Km is effectively independent of temperature. On the other hand, the high Km value at 0°C is what one might expect if the enzyme proved less stable at lower temperatures than the preparation from 4°C-acclimated crayfish.

DISCUSSION

A. Activity Requirements of the Enzyme

The Mg^{2+}-dependent ATPase of sarcoplasmic reticulum fragments was inhibited by low concentrations of EGTA. Addition of low concentrations of calcium to the reaction medium caused a marked stimulation of ATPase activity. This indicates calcium contamination of the reaction medium by the enzyme preparation, the ionic medium or possibly from the ATP solution (Martonosi and Feretos, 1964). Although commercial ATP is known to be a potential source of calcium contamination (1 mole of calcium per 500 moles of ATP; Seidel and Gergely, 1963), the treatment with 'Dowex' $50H^{+}$ resin would ensure a calcium-free ATP solution (Seidel and Gergely, 1963). It is significant

that Sreter (1969) has reported, in rabbit SR purified by continuous sucrose density gradient, an endogenous calcium content of 13.8 $\pm$ 0.3 moles per mg. of protein. 0.5uM EGTA was found to cause maximal suppression of ATP hydrolysis in the absence of calcium and all subsequent assays were performed at this concentration of EGTA.

The complex activation-inhibition kinetics and the free Ca^{2+} concentration required for maximal Ca^{2+}-Mg^{2+}-ATPase activity for crayfish fragmented sarcoplasmic reticulum, are in good agreement with other published work, using the Ca^{2+}-Mg^{2+}-ATPase from mammalian skeletal muscle microsomes (Martonosi and Feretos,1964; Hasselbach, 1964; Yamamoto and Tonomura,1967), mammalian skeletal muscle sarcolemma (Sulakhe, Drummond and Ng,1973a), bovine brain microsomes (optimal free Ca^{2+} = 65uM, Roufogalis,1973). Similar Ca^{2+} optima have been reported for calcium-uptake measurements in frog skeletal muscle sarcoplasmic reticulum (Hasselbach,1964). All of these preparations show maximal calcium-activation with approximately 1uM to 10uM free Ca^{2+}. The cause of Ca^{2+}-induced inhibition at calcium concentrations above pCa 5 is not clear but may be caused by competition between calcium and magnesium for ATP chelation, resulting in a lowered Mg-ATP concentration in the reaction medium, for it is suggested that the substrate for these ATPases is a Mg^{2+}-ATP complex.

The calcium activation-inhibition characteristics and optimal free Ca^{2+} concentration of microsomal preparations isolated from both 4°C and 25°C-acclimated crayfish were identical. The absence of any acclimation effect in this respect suggests that Ca^{2+} flux during contraction and relaxation in muscle is not affected by adaptation to different environmental temperatures.

The absence of a significant Ca^{2+}-dependent ATPase activity in the preparation and the insensitivity of the Ca^{2+}-stimulated Mg^{2+}-ATPase to sodium azide, a specific inhibitor of the mitochondrial calcium pump (Fanberg and Gergely,1965) confirmed that at least 95% of the Ca^{2+}-activated, ATP hydrolysis was due to the stimulation of a Mg^{2+}-dependent system and not due to other calcium-activated systems (Weber, Herz and Reiss, 1966).

B. Saturation Kinetics

There are conflicting reports in the literature concerning the saturation kinetics with respect to ATP, of the fragmented sarcoplasmic reticulum (FSR). On one hand, several workers have discovered biphasic saturation curves for the Ca^{2+}-Mg^{2+}-ATPase (Yamamoto and Tonomura,1967; Inesi, Goodman and Watanabe,1967; Horgan,1974), the phosphorylation of membranous protein (Inesi,1970) and the Ca^{2+}-uptake of rabbit skeletal muscle FSR (Inesi,1967; Weber, Herz and Reiss,1966) similar to those reported here (see Table 6-12). In contrast, other workers report simple Michaelis-Menten kinetics for the same systems (Panet, Pick and Selinger,1971; Sulakhe, Drummond and Ng, 1973a; Walter and Hasselbach,1973). Furthermore, there are conspicuous differences in the values of Km reported by these workers (see Table 6-12). The reasons for these conflicting results are not clear, but do not appear to be related to the methods of preparation or enzyme assay. It was unfortunate that reaction velocity measurements were not possible at substrate concentrations less than 0.1mM, since the values of Km for high affinity (low Km) activity reported in all publications lay below this concentration (Table 6-12). However, the projected lines for the Lineweaver-Burk plots are in good agreement with the

data reported here. There is no suggestion of a catenary curve between 50uM and 5mM ATP as reported by Horgan (1974).

There are two alternatives to be considered in explaining the biphasic Lineweaver-Burk plots. Firstly, there may be two different Ca^{2+}-activated, Mg^{2+}-dependent ATPases operating within the system, each with very different Km's such that each enzyme becomes saturated at very different concentrations of ATP (Dixon and Webb,1964; Ruiz-Herrera, Alvarez and Figueroa,1972). This situation could be confirmed only with the chromatographic or electrophoretic demonstration of two separate enzymes.

The alternative explanation is that the same enzyme is responsible for each type of activity, and that there is some change in the kinetic parameters of the system in response to different concentrations of ATP. All available evidence favours this latter suggestion since many kinetic propreties of each type of activity are very similar, if not identical. For example, Yamamoto and Tonomura (1967) have found that the dependencies of high and low affinity ATPase activity on temperature, pH, the Ca^{2+} uptake/ATP hydrolysis ratio and N-ethyl maleimide inhibition, were remarkably similar. In the present investigation the mean Arrhenius activation energy at high and low affinity were identical (see Chapter 7). Horgan (1974) has recently demonstrated that it is possible to obtain a simple linear Lineweaver-Burk plot by maintaining an equimolar mixture of Mg^{2+} and ATP over the entire range of ATP concentrations. This linear plot had a slope that was in between that for high (ATP) activity and that for low (ATP) activity.

The modified kinetic properties of the FSR system in response to a change ATP concentration may be due to a direct effect of ATP upon the enzyme regulatory machinery. ATP would act, therefore, upon the enzyme not only as a substrate but also as a regulator which controls the binding of substrate and rate of decomposition of the enzyme-substrate complex (Yamamoto and Tonomura,1967). It is interesting that similar concentrations of ATP (in the absence of Mg^{2+}) are known to cause changes in the electron paramagnetic resonance spectrum of rabbit skeletal FSR, indicating some conformational change due to simple binding of ATP to the preparation (Landgraf and Inesi 1969).

Alternatively, ATP may have an indirect effect upon the rate of ATP hydrolysis by the sarcoplasmic vesicles. In this context it is possible that free ATP may penetrate into the vesicles and complex with accumulated Ca^{2+}, decreasing the intravesicular Ca^{2+} concentration sufficiently to release the enzyme from this inhibitory influence (Martonosi,1972). This scheme is consistent with the observation by Weber (1971) that the inhibitory effect of accumulated calcium on steady-state calcium flux is alleviated by relatively high concentrations of free ATP (not bound to Mg^{2+}). Other factors to be considered include the suggestion that the presence of hydrolysis products of ATP can stimulate the rate of efflux from the vesicles (Barlogie, Hasselbach and Makinose,1971, Panet and Selinger,1972; Masuda and De Meis,1974)releasing the inhibition of Ca^{2+}-uptake activity caused by accumulated calcium.

In the absence of further data it is not possible to make firm conclusions regarding the physiological significance of each type of activity. It is possible

however, to see some physiological advantage in possessing a system, which is capable of increasing its activity to a significantly higher level at low substrate concentration than one would predict from its behaviour at higher substrate concentrations. Unfortunately, the *in vivo* concentration and distribution of ATP is not known although Gladwell (1973) has estimated the overall ATP content of muscles to be 1.3uM per gram wet muscle weight which approximates to a bulk concentration of 1mMolar. However, the distribution of this ATP within the highly compartmentalised muscle cell is totally unknown, and we can only guess at the concentration of ATP experienced by the enzyme (Bygrave 1967). For this reason kinetic analysis of the Ca^{2+}-Mg^{2+}-ATPase was performed at both high and low concentrations of ATP.

C. The Effect of Temperature and Thermal Acclimation on the E-S Affinity

The analysis of Km values presented here indicates that for velocity measurements with an appreciable error, the value of Km determined by graphical means and regression analysis is subject to a degree of uncertainty. For this reason it was not considered valid to quote a single value of Km, and one should give some indication of the likely error associated with each Km determination. Only then is it possible to compare the Km's of different preparations.

Consideration of the results illustrated in Figures 6-14 and 6-15 has led to the conclusion that the Km for ATP at high and low [ATP] activity is not clearly dependent upon temperature. It was concluded that the enzyme under consideration, therefore, did not possess an automatic mechanism for maintaining its activity at least partially independent of rapid, short term temperature fluctuations,

as has been observed in several "soluble" (Hochachka and Somero,1973), mitochondrial (Hazel,1972) and other membrane-bound enzymes (Berlin,1973). Neither is there any evidence to suggest the existence of a minimal Km value in the normal temperature range of the crayfish as has been reported for various 'soluble' enzyme systems (Hochachka and Somero,1973). Furthermore, acclimation of crayfish to different environmental temperatures caused no change in the Km/temperature characteristics of the Ca^{2+}-Mg^{2+} ATPase. This conclusion implies that the same protein species was present in both acclimation groups.

The significance of the large increase in Km (ATP) for Ca^{2+}-Mg^{2+}-ATPase at 35°C in both acclimation groups is unknown. It should be borne in mind that this temperature is lethal to the crayfish and so the increase in Km may represent a direct effect of temperature upon the enzyme since, as discussed previously, the temperature dependence of the kinetic parameters of an enzyme will be determined in large part by the effect of temperature on the weak bonds that are responsible for its conformational state. Alternatively,it may indirectly reflect some change in the physical state of the lipid environment surrounding the enzyme. Gladwell (1973) has demonstrated a marked decline, with time, of the membrane resistance of crayfish sarcolemma at the elevated temperatures that cause heat death (Bowler, Duncan, Gladwell and Davison, 1973). He interpreted this as an increase in membrane permeability due to heat stress. This may well be caused by increased micro-viscosity or "fluidity" of the hydrocarbon interior of the membrane resulting in an increased leakiness to electrolytes. A similar increase in temperature and membrane fluidity of the membrane environment may have deleterious effects on the physical state and catalytic efficiency of membrane enzymes.

Table 6-1: Comparison of the average yield of microsomal protein from the abdominal muscle of $4^{o}C$ and $25^{o}C$ acclimated crayfish.

Extraction Procedure:

See Chapter 5.

Protein Analysis: The protein content of microsomal preparations was estimated by the Folin-Ciocalteau method of Lowry, Rosebrough, Farr and Randall (1951). See Chapter 5.

Legend:

- n' - number of different preparations assayed for each acclimation group.
- P - Probability for (n-2) degrees of freedom.
- V.S. - Very significant.

Acclimation Temperature ^{o}C	4	25
Average Yield (mg.protein/gm wet muscle wt.)	0.643	0.897
S.E.M.	0.098	0.246
n	12	8
t	3.622	
P	0.01 - 0.001	
Significance	V.S.	

Table 6-2: The effect of Ca^{2+}, Mg^{2+} and sodium azide upon ATP hydrolysis by a microsomal preparation extracted from the muscle of 4°C acclimated and from the muscle of 25°C acclimated crayfish.

For the composition of assay media see Chapter 5. Values reported are the means of duplicate analyses performed on one microsomal preparation from each acclimation group.

Legend:

* % of Total Ca^{2+}-Mg^{2+}-ATPase activity.

Acclimation Temperature ^{o}C		4	25
Assay Temperature ^{o}C		25	25
ATPase Activity (uM Pi. mg protein^{-1}. minute^{-1}.)	Total Ca^{2+}-Mg^{2+} ATPase (Ca^{2+}, Mg^{2+})	0.716	0.429
	Ca^{2+}-stimulated ATPase	0.673 (94.0%)*	0.424 (98.8%)*
	Mg^{2+}-dependent ATPase (Mg^{2+})	0.043 (6.0%)*	0.005 (1.2%)*
	Ca^{2+}-dependent ATPase (Ca^{2+})	0.000 (-)*	0.000 (-)*
	Total Ca^{2+}-Mg^{2+} ATPase with 5mM Sodium Azide (Ca^{2+}, Mg^{2+}, Azide)	0.724 (101.1%)*	0.413 (96.4%)*

Table 6-3 Summary of the free $[Ca^{2+}]$ concentration required for optimal stimulation of Mg^{2+}-dependent ATPase activity at different temperatures, and the % error in ATPase activity measurement caused by assay with 8.1 uM free $[Ca^{2+}]$ and not the true optimal free $[Ca^{2+}]$

The values of optimal free $[Ca^{2+}]$ at each assay temperature were determined from Figures 6-5 and 6-6. The "% error in enzyme activity" (*) was the difference between the maximal enzyme activity, and activity in the presence of 8.1 uM free calcium, expressed as a percentage of the maximal enzyme activity.

Assay Temperature	Acclimation Temperature			
	4°C		25°C	
°C	Optimal free $[Ca^{2+}]$	% Error in Activity assay*	Optimal free $[Ca^{2+}]$	% Error in Activity assay*
5.0	14.0	- 2.1	13.5	- 1.9
20.0	7.0	0.0	7.6	0.0
35.0	2.8	- 2.7	2.9	- 2.1

Table 6-4: The Ca^{2+}-stimulated ATPase activity of a microsomal preparation isolated from $4^{o}C$ acclimated crayfish muscle, measured at different ATP concentrations and at temperatures between $0^{o}C$ and $35^{o}C$.

Extraction Procedure: The abdominal muscle from seven crayfish acclimated to $4^{o}C$ was extracted as described in Chapter 5. The preparation was stored at $-20^{o}C$ until used.

Assay Conditions: The Ca^{2+}-stimulated ATPase was assayed as described in Chapter 5, at different ATP concentrations for each assay temperature (see 'Methods').
All temperatures are $\pm 0.1^{o}C$.

Phosphate and Protein Analysis:
Described in Chapter 5.

Concentration of ATP (mM)	Activity (uM Pi. mg protein^{-1}. minute^{-1}.)							
	0°C	4°C	8°C	12°C	18°C	23°C	28°C	35°C
3.00	0.089	0.148	0.232	0.279	0.541	1.032	1.266	2.577
1.50	0.078	0.127	0.191	0.264	0.522	0.830	1.068	2.400
1.00	0.070	0.109	0.187	0.230	0.450	0.740	0.982	2.232
0.50	0.056	0.092	0.153	0.206	0.399	0.585	0.815	1.903
0.50	0.059	0.088	0.152	0.198	0.431	0.588	0.840	1.782
0.30	0.051	0.080	0.148	0.181	0.388	0.551	0.784	1.686
0.20	0.052	0.068	0.128	0.153	0.318	0.514	0.673	1.469
0.20	0.050	0.068	0.116	0.163	0.321	0.496	0.679	1.381
0.16	0.041	0.067	0.115	0.145	0.321	0.454	0.630	1.317
0.13	0.047	0.062	0.112	0.140	0.297	0.430	0.661	1.196
0.10	0.037	0.058	0.103	0.149	0.273	0.359	0.537	0.987
0.10	0.037	0.047	0.089	0.129	0.265	0.390	0.549	0.963

Table 6-5: The Ca^{2+}-stimulated ATPase activity of a microsomal preparation isolated from 25°C acclimated crayfish muscle measured at different ATP concentrations for each assay temperature between 0°C and 35°C.

Extraction Procedure:- The abdominal muscle from seven crayfish acclimated to 25°C was extracted as described in Chapter 5. The microsomal preparation was stored at -20°C until required.

Assay Conditions:- The Ca^{2+}-stimulated ATPase was assayed as described in Chapter 5 at different ATP concentrations for each assay temperature (see 'Methods').
All temperatures are $\pm$0.1°C.

Phosphate and Protein Analysis:- As described in Chapter 5.

Concentration of ATP (mM)	Activity (uM Pi . mg protein^{-1}. minute^{-1}.)							
	0°C	4°C	8°C	12°C	18°C	23°C	28°C	35°C
3.00	0.114	0.182	0.228	0.341	0.555	0.781	1.209	3.026
1.50	0.096	0.144	0.212	0.279	0.487	0.711	1.080	2.456
1.00	0.084	0.129	0.175	0.267	0.447	0.628	0.889	2.094
0.50	0.059	0.115	0.154	-	0.425	0.604	0.744	2.059
0.30	0.055	0.097	-	0.209	0.357	0.509	0.775	1.650
0.20	0.051	0.090	0.134	0.186	0.295	0.496	0.605	1.588
0.20	0.055	0.086	0.135	0.188	0.300	0.480	0.605	1.563
0.16	0.048	0.084	0.124	0.174	0.295	0.424	0.568	1.464
0.13	0.048	0.079	0.120	0.167	0.284	0.413	0.517	1.289
0.12	0.047	0.075	0.114	0.161	0.264	0.409	0.584	1.278
0.10	0.047	0.069	0.109	0.151	0.264	0.376	0.543	1.116
0.10	0.037	0.067	0.105	0.155	0.253	0.401	0.475	1.166

Table 6-6: The results of regression analysis of the Lineweaver-Burk plots and Eadie-Hofstee plots for high [ATP] activity of the Ca^{2+}-stimulated ATPase activity of a single muscle microsomal preparation isolated from $4^{o}C$ acclimated crayfish

For details of extraction and analysis of Ca^{2+}-stimulated ATPase activity, see text. Ca^{2+}-stimulated ATPase was assayed in the presence of 0.5 - 3.0mM ATP. The basic data is presented in Table 6-4, and the techniques used for the calculation of the kinetic constants Km and Vmax are reported in 'Materials and Methods'.

Legend:

$\overline{X}$	=	$^{1}/Km$
S.E.M*	-	Standard Error of $\overline{X}$.
S.E.M$^{\neq}$	-	Standard Error of Km (Eadie-Hofstee plot)
Vmax	-	(uM Pi liberated. mg protein^{-1}. minute^{-1}.)
n	-	Number of enzyme assays at each temperature
P	-	Level of significance of correlation coefficient.

Acclimation Temperature °C	Assay Temperature °C	n	Lineweaver-Burk Plot						Eadie-Hofstee Plot		
			r	P	Vmax	$\overline{X}$	S.E.M*	Km (uM)	Km (uM)	S.E.M. ≠	Vmax
4	0	5	0.9864	<0.01	0.097	2.84	0.42	351	412	172	0.102
	4	5	0.9857	<0.01	0.161	2.53	0.40	403	448	98	0.167
	8	5	0.9812	<0.01	0.242	3.36	0.70	297	312	10	0.246
	12	5	0.9794	<0.01	0.299	4.09	0.68	244	255	14	0.301
	18	5	0.9339	<0.05	0.569	5.21	0.49	192	189	46	0.568
	23	5	0.9907	<0.01	1.123	2.15	0.50	464	490	65	1.145
	28	5	0.9829	<0.01	1.326	3.24	0.52	308	319	48	1.341
	35	5	0.9908	<0.01	2.815	3.78	0.43	264	259	28	2.803

Table 6-7: The Results of regression analysis of the Lineweaver-Burk plots and Eadie-Hofstee plots for low [ATP] activity of the Ca^{2+}-stimulated ATPase activity of a single muscle microsomal preparation isolated from $4^{o}C$ acclimated crayfish.

For details of extraction and analysis of Ca^{2+}-stimulated ATPase activity, see text. Ca^{2+}-stimulated ATPase activity was assayed in the presence of 0.1 - 0.5mM ATP. The basic data is presented in Table 6-4, and the techniques used for the calculation of the kinetic constants Km and Vmax are reported in 'Materials and Methods'.

Legend:

$\bar{X} = {}^{1}/_{Km}$

S.E.M.* - Standard Error of $\bar{X}$ (Lineweaver-Burk plot)

S.E.M.$^{\neq}$ - Standard Error of Km (Eadie-Hofstee plot)

Vmax - (uM Pi liberated. mg protein^{-1}. minute^{-1}.)

n - Number of enzyme assays at each temperature.

P - Level of significance of correlation coefficient.

Acclimation Temperature °C	Assay Temperature °C	n	Lineweaver-Burk Plot						Eadie-Hofstee Plot		
			r	P	Vmax	$\bar{X}$	S.E.M*	Km (uM)	Km (uM)	S.E.M. ≠	Vmax
4	0	9	0.9258	<0.001	0.067	12.99	3.21	76.9	70.3	14.1	0.065
	4	8	0.9731	<0.001	0.101	12.29	1.85	81.3	93.7	10.0	0.104
	8	9	0.9504	<0.001	0.181	11.30	2.39	88.5	82.5	10.0	0.177
	12	8	0.9783	<0.001	0.229	12.03	1.64	83.1	61.2	10.0	0.215
	18	9	0.9592	<0.001	0.461	13.25	2.40	75.5	76.7	13.0	0.446
	23	9	0.9833	<0.001	0.704	11.59	1.39	86.3	80.9	12.1	0.691
	28	9	0.9708	<0.001	0.943	13.95	2.06	71.7	69.1	10.0	0.935
	35	9	0.9918	<0.001	2.464	6.75	0.79	148.1	133.3	10.0	2.355

Table 6-8: The results of regression analysis of the Lineweaver-Burk plots and Eadie-Hofstee plots for high [ATP] activity of the Ca^{2+}-stimulated ATPase activity of the muscle microsomal preparation isolated from 25°C acclimated crayfish.

For details of extraction and analysis of Ca^{2+}-stimulated ATPase activity, see text. Ca^{2+}-stimulated ATPase was assayed in the presence of 0.5mM - 3.0mM ATP. The basic data is presented in Table 6-5, and the techniques used for the calculation of the kinetic constants Km and Vmax are reported in 'Materials and Methods'.

Legend:

$\bar{X}$ = $1/Km$

S.E.M.* - Standard Error of $\bar{X}$ (Lineweaver-Burk plot)

S.E.M.$^{\neq}$ - Standard Error of Km (Eadie-Hofstee plot)

Vmax - (uM Pi. liberated. mg protein^{-1}. minute^{-1}.)

n - Number of enzyme assays at each temperature

P - Level of significance of correlation coefficient

Acclimation Temperature °C	Assay Temperature °C	n	Lineweaver-Burk Plot						Eadie-Hofstee Plot		
			r	P	Vmax	$\bar{X}$	S.E.M. *	Km (uM)	Km (uM)	S.E.M. ≠	Vmax
25	0	4	1.000	<0.001	0.140	1.48	0.012	674	675	14	0.147
	4	4	0.9281	<0.1	0.183	3.13	1.24	319	351	110	0.187
	8	4	0.9628	<0.1	0.247	3.17	0.87	316	334	45	0.251
	12	3	0.9475	<0.1	0.385	2.11	0.96	473	509	43	0.392
	18	4	0.9002	<0.1	0.552	6.03	2.46	166	174	67	0.557
	23	4	0.8992	>0.1	0.787	5.94	2.44	168	174	66	0.793
	28	4	0.9795	<0.05	1.343	2.39	0.54	417	433	88	1.359
	35	4	0.8346	>0.1	2.927	4.09	2.47	244	382	150	2.980

Table 6-9: The results of regression analysis of the Lineweaver-Burk plots and Eadie-Hofstee plots for low [ATP] activity of the Ca^{2+}-stimulated ATPase activity of the muscle microsomal preparation isolated from 25°C acclimated crayfish.

For details of extraction and analysis of the Ca^{2+}-stimulated ATPase activity, see text. Ca^{2+}-stimulated ATPase activity was assayed in the presence of 0.1mM - 0.5mM ATP. The basic data is presented in Table 6-5, and the techniques used for the calculation of the kinetic constants Km and Vmax are reported in 'Materials and Methods'.

Legend:

$\bar{X}$ = $^{1}/_{Km}$

S.E.M.* - Standard error of $\bar{X}$ (Lineweaver-Burk plot)

S.E.M.≠ - Standard error of Km (Eadie-Hofstee plot)

Vmax - (uM Pi liberated. mg protein^{-1}. minute^{-1}.)

n - Number of Ca^{2+}-stimulated activity determinations at each temperature

P - Level of significance of the correlation coefficient.

Acclimation Temperature °C	Assay Temperature °C	n	Lineweaver-Burk Plot						Eadie-Hofstee Plot		
			r	P	Vmax	$\bar{X}$	S.E.M. *	Km (uM)	Km (uM)	S.E.M. ≠	Vmax
25	0	10	0.8723	<0.001	0.066	20.52	1.99	48.7	52.9	68.0	0.122
	4	9	0.9923	<0.001	0.132	10.78	0.48	92.8	92.6	141.0	0.131
	8	8	0.9918	<0.001	0.176	15.74	1.25	63.6	62.7	10.0	0.175
	12	8	0.9904	<0.001	0.246	13.51	1.39	74.0	63.9	10.0	0.248
	18	9	0.9425	<0.001	0.440	13.42	2.89	74.5	79.1	14.1	0.448
	23	9	0.9637	<0.001	0.651	14.09	2.55	71.0	72.3	10.1	0.656
	28	9	0.8858	<0.01	0.848	14.42	4.45	69.4	59.6	10.1	0.817
	35	9	0.9887	<0.001	2.424	8.99	1.09	111.3	110.0	10.0	2.416

Table 6-10: Comparison of the mean Km values for high and low [ATP] activity in the Ca^{2+}-stimulated ATPase activity of the microsomal preparations from both 4°C and 25°C acclimated crayfish.

Assay Conditions:- The Ca^{2+}-stimulated ATPase of each microsomal preparation was assayed at different ATP concentrations between 0.1mM and 3.0mM, at various temperatures between 0-35°C (see 'Methods' and Tables 6-6 to 6-9). Vmax and Km were calculated from the 'best fit' line of this data, as estimated by regression analysis (Snedecor and Cochran, 1967; see 'Methods').
The mean Km was calculated for determinations at temperatures from 0°C to 28°C for low [ATP]activity(*), and from 0°C to 35°C for high [ATP] activity($\neq$).

Phosphate and Protein Analysis:- As described in Chapter 5.

Legend:-

n	-	number of Km determinations
P	-	probability for (n-2) degrees of freedom
N.S.	-	not significant
S.E.M.	-	standard error of the mean

Range of ATP concentrations	Low * (0.1-0.5mM ATP)		High ≠ (0.5-3.0mM ATP)	
Acclimation Temperature ^{o}C	4	25	4	25
Mean Km (uM)	80.47	70.57	315.4	347.5
S.E.M.	2.29	4.99	31.2	60.5
n	7	7	8	8
t	1.70		0.472	
P	$<$0.20		$<$0.70	
Significance	N.S.		N.S.	

Table 6-11: Comparison of the Km (for Low [ATP] activity) at 35°C, with the mean Km (Low [ATP] activity) for lower temperatures, for microsomal preparations isolated from both 4°C and 25°C acclimated crayfish.

Assay Conditions:- The Km for each preparation was determined as described in the text, at temperatures between 0°C and 35°C. The Km's for low [ATP] activity for temperatures between 0°C and 28°C were averaged (see Tables 6-7 and 6-9).

Phosphate and Protein Analysis:- As described in Chapter 5.

Legend:-

*	-	Difference between Km at 35°C and the mean Km for temperatures below 28°C, in units of standard deviation of the mean Km.
P	-	Probability for (n-1) degrees of freedom.
H.S.	-	Highly significant.
V.S.	-	Very significant.

Acclimation Temperature (^{o}C)	4	25
Km at 35^{o}C (uM)	148.1	111.0
Difference with mean Km (S.D's)*	11.16	3.09
P	<0.001	<0.01
Significance	H.S.	V.S.

Table 6-12: Summary of data from the literature concerning the biphasic saturation characteristics of the Ca^{2+}-stimulated ATPase isolated from various tissues.

* Calculated from Yamamoto and Tonomura (1967).

? Data not provided by authors.

Source of Microsomal Preparation	Activity Monitored	Km (uM)	Vmax (uM Pi . mg protein^{-1}.mm^{-1}.)	Reference
Rabbit White Skeletal Muscle	Ca^{2+}-stimulated ATPase [ATP]	0.7 30	1.14* 3.0 *	Yamamoto and Tonomura (1967)
Rabbit White Skeletal Muscle	Phosphorylation of sarcoplasmic reticulum membrane [P_{32}]	1.6 ?	? ?	Inesi, Maring, Murphy and MacFarland (1970)
Rabbit White Skeletal Muscle	Ca^{2+}-stimulated ATPase [ATP]	10 1000	1.7 0.6	Inesi, Goodman and Watanabe (1967)
Human Erythrocyte Membrane	Ca^{2+} ATPase [Ca^{2+}]	4 100	_ 66% _ 33%	Schatzmann and Rossi (1971)
Crayfish Abdominal Muscle	Ca^{2+}-stimulated ATPase [ATP]	70-80 315-347	_ 66% _ 33%	Present work
Rabbit White Skeletal Muscle	Ca^{2+}-stimulated ATPase [ATP]	1.0 ?	? ?	Horgan (1974)

Figure 6-2 The effect of increasing concentration of EGTA upon the 'Basal' Mg^{2+}- dependent ATPase activity of a muscle microsomal preparation extracted from $4^{o}C$ acclimated crayfish.

Assay Conditions: ATP hydrolysis was measured at $25^{o}C$ in the presence of different concentrations of EGTA, in a medium containing 3mM $MgCl_2$, 3mM ATP, 100mM KCl, 25mM Imidazole - HCl, pH 7.1.

Legend: Ordinate - ATPase activity (uM Pi liberated, mg $protein^{-1}$. $minute^{-1}$.)

Abscissa - mM EGTA in reaction medium.

(uM Pi.mgprotein⁻¹.minutes⁻¹)
1.0
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
1.0
EGTA(mM)

Figure 6-3 The effect of different concentrations of free Ca^{2+} upon the activity of the Mg^{2+}-dependent ATPase of a muscle microsomal preparation isolated from $4^{o}C$ acclimated crayfish.

Assay Conditions: The reaction medium contained 0.5mM EGTA 3mM $MgCl_2$, 100mM KCl, 25mM Imidazole - HCl, pH 7.1, and various concentrations of $CaCl_2$. The free calcium concentration in the reaction medium was contolled using EGTA as discussed in 'Material and Methods'.

Values plotted are the stimulation of ATP Hydrolysis above that in the above reaction medium but omitting $CaCl_2$.

Incubation temperature was $20^{o}C$.

Legend: Ordinate - ATPase activity (uM Pi liberated. mg $protein^{-1}$. $minute^{-1}$.)

Abscissa - Free calcium concentration, pCa (M).

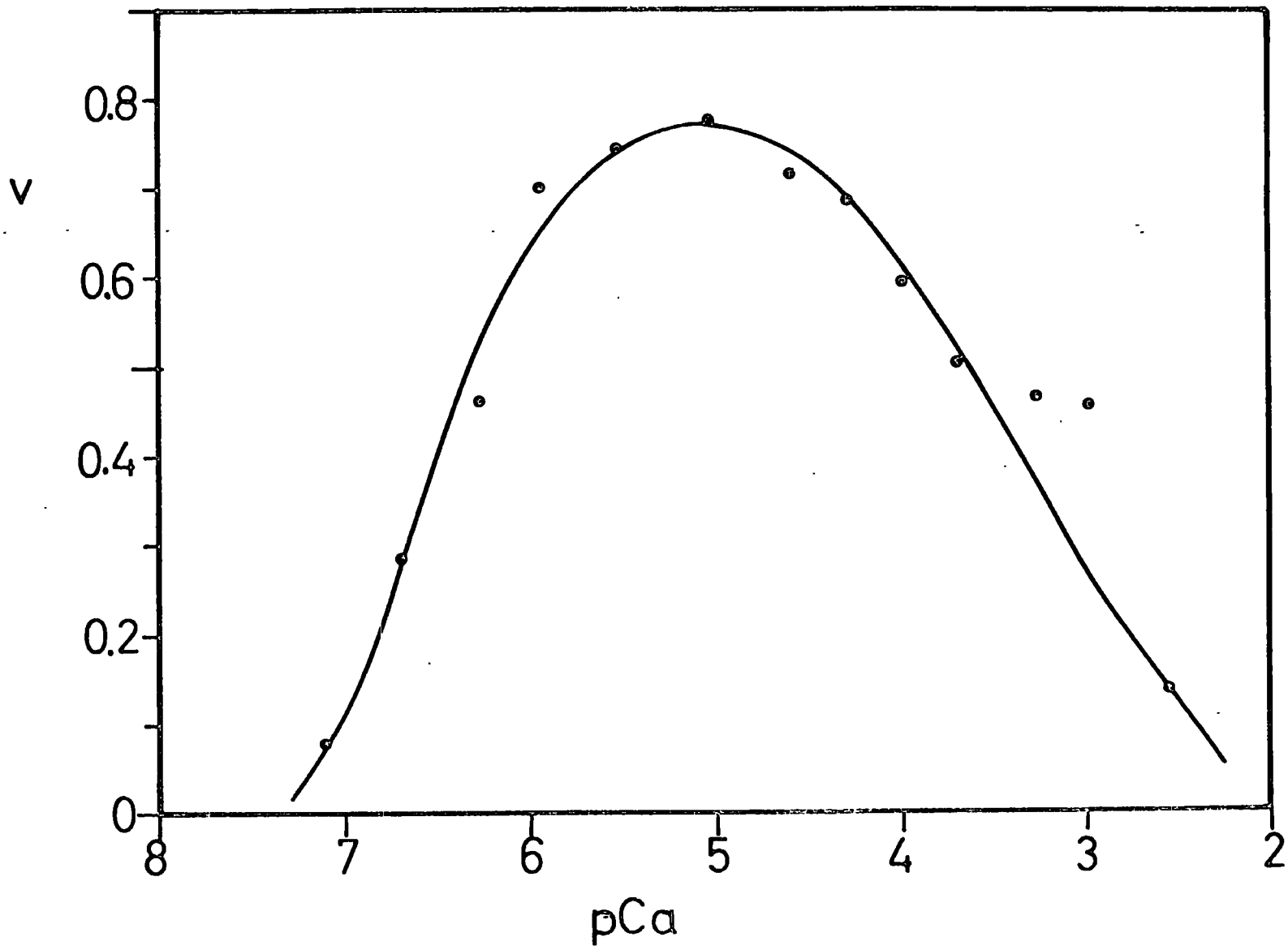

v
0.8
0.6
0.4
0.2
0
8
7
6
5
4
3
2
pCa

Figure 6-4: The time-course of ATP hydrolysis at 5°C, 20°C and 35°C in the presence of 3.0mM ATP, by a microsomal Ca^{2+}-stimulated ATPase isolated from 4°C acclimated crayfish.

Assay Conditions: 'Total' Ca^{2+}-Mg^{2+}-ATPase activity was determined in the presence of 0.5mM $CaCl_2$, 0.5mM EGTA, 3mM $MgCl_2$, 75mM Sucrose, 100mM KCl, 25mM Imidazole -HCl pH 7.1 and 3.0mM ATP. 'Basal' Mg^{2+} ATPase was assayed in the same medium but omitting $CaCl_2$. Values plotted (Ca^{2+}-stimulated ATPase) were the difference between 'Total' and 'Basal' ATP hydrolysis for each incubation period at each temperature. For other details see 'Materials and Methods'.

Legend: Ordinate - ATP hydrolysis (uM Pi liberated. mg protein^{-1}.)

Abscissa - Period of Incubation (minutes).

□ - 5°C Incubation Temperature

○ - 20°C " "

▲ - 35°C " "

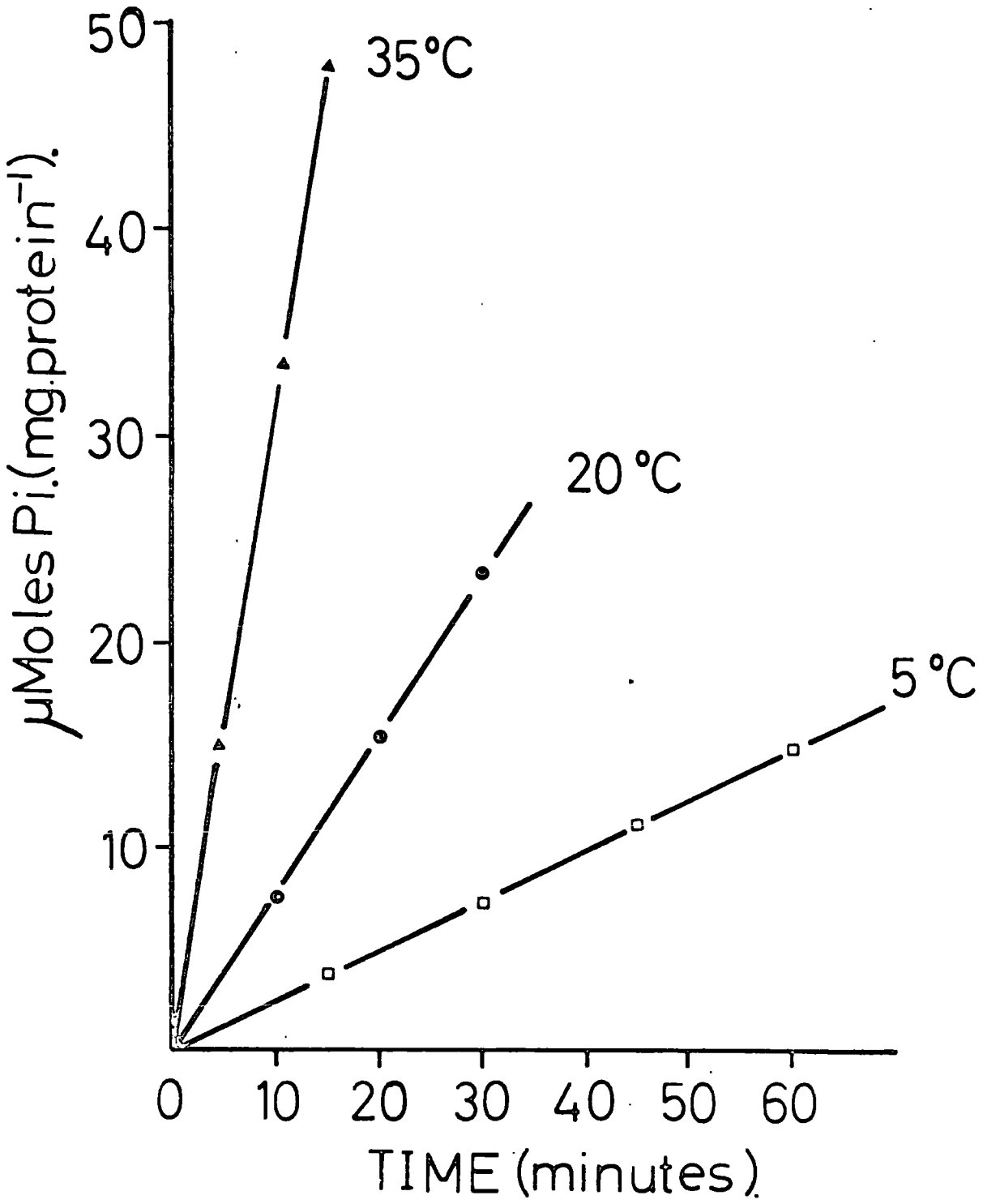

50
40
30
20
10
µMoles Pi.(mg.protein⁻¹).
35°C
20°C
5°C
0
10
20
30
40
50
60
TIME (minutes)

Figure 6-5 The effect of different incubation temperatures upon the Ca^{2+}- activation and inhibition characteristics of the Mg^{2+}- dependent ATPase of a microsomal preparation isolated from $4^{o}C$ acclimated crayfish.

Assay Conditions: The reaction media are described in 'Materials and Methods'. The activation/inhibition characteristics of the Mg^{2+}- dependent ATPase were measured as described in Figure 6-2 at $5^{o}C$, $20^{o}C$ and $35^{o}C$.

Legend: Ordinate - ATPase activity (uM Pi liberated. mg protein^{-1}. minute^{-1}.)

Abscissa - Concentration of free Ca^{2+}, pCa (M).

▼ - $5^{o}C$ Incubation Temperature

O - $20^{o}C$ " "

o - $35^{o}C$ " "

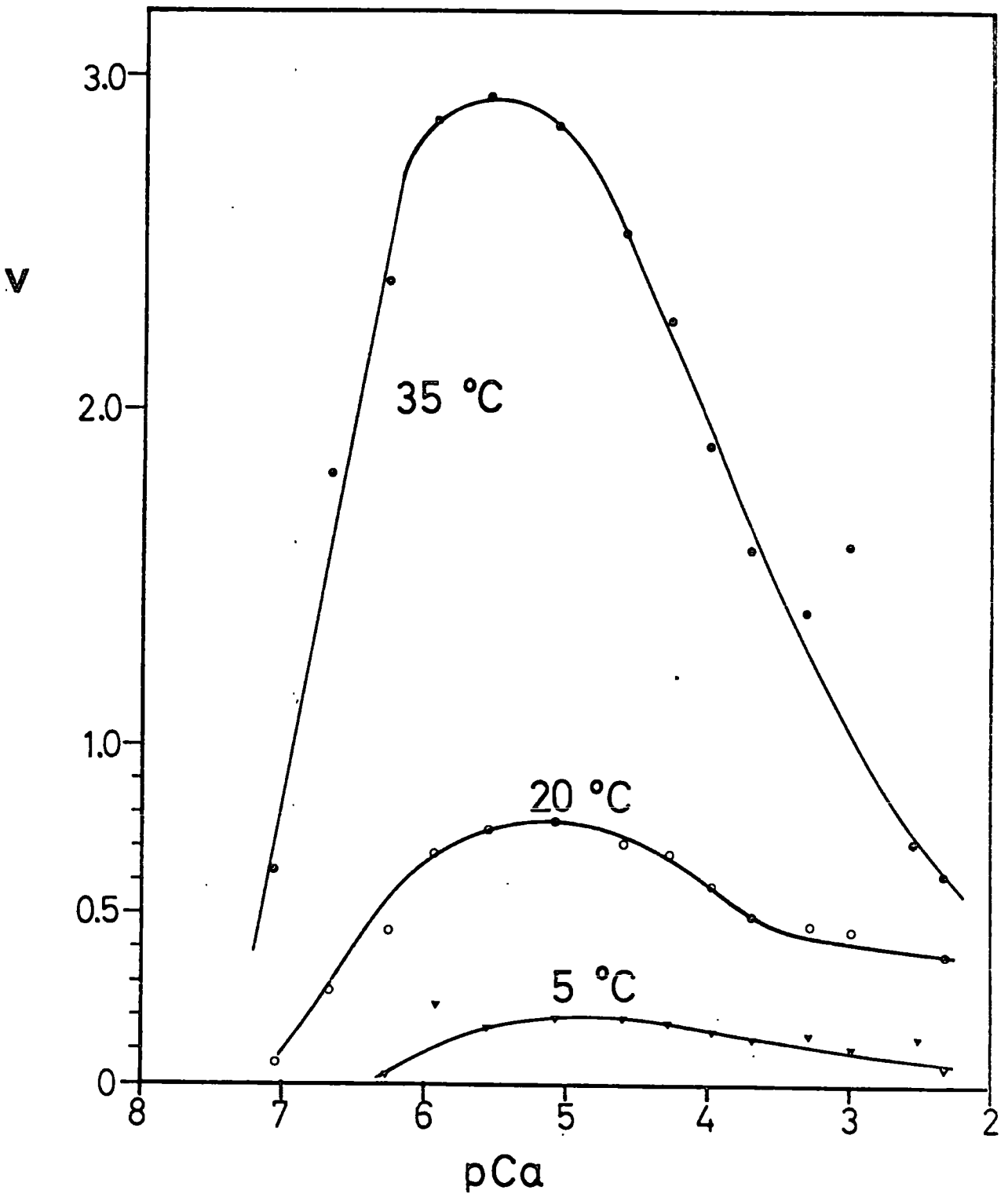

V
3.0
2.0
1.0
0.5
0
35 °C
20 °C
5 °C
8
7
6
5
4
3
2
pCa

Figure 6-6 The effect of different incubation temperatures upon the Ca^{2+}- activation and inhibition characteristics of the Mg^{2+}-dependent ATPase of a microsomal preparation isolated from 25°C acclimated crayfish.

Assay Conditions: The reaction media are described in 'Materials and Methods'. The activation/inhibition characteristics of the Mg^{2+}- dependent ATPase were measured as described in Figure 6-2 at 5°C, 20°C and 35°C.

Legend: Ordinate - ATPase activity (uM Pi liberated. mg $protein^{-1}$. $protein^{-1}$.)

Abscissa - Concentration of free Ca^{2+}, pCa (M).

▼ - 5°C Incubation Temperature

○ - 20°C " "

● - 35°C " "

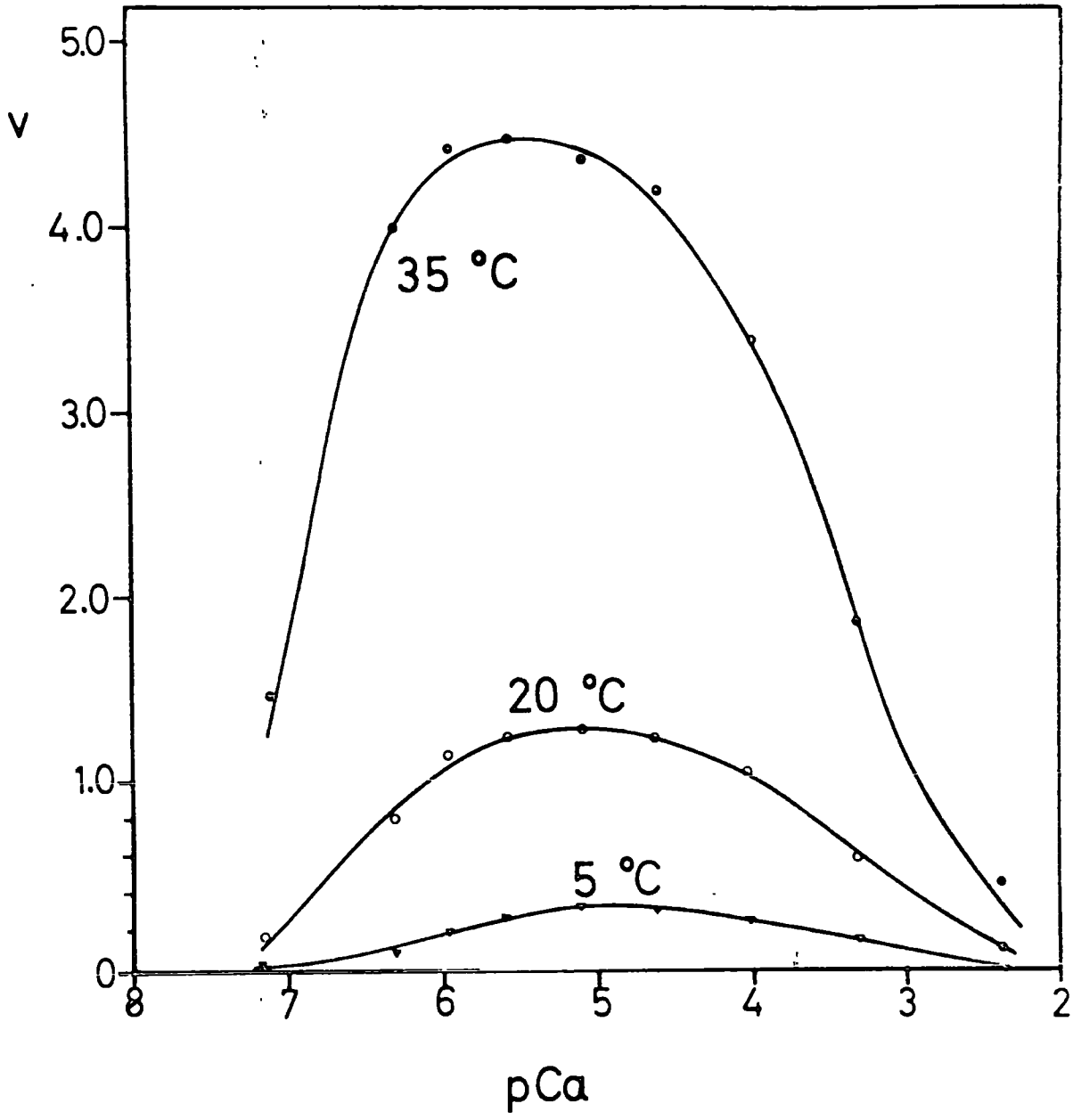

V
5.0
4.0
3.0
2.0
1.0
0
35 °C
20 °C
5 °C
8
7
6
5
4
3
2
pCa

Figure 6-7a The effect of incubation temperature upon the free Ca^{2+} concentration required for optimal Ca^{2+}- stimulated ATPase activity.

Assay Conditions: The optimal free Ca^{2+} concentrations at each incubation temperature were determined from Figures 6-5 and 6-6.

Legend: Ordinate - Optimal free Ca^{2+} concentration (uM)

Abscissa - Incubation temperature (^{o}C)

● - Microsomal preparation from $4^{o}C$ acclimated crayfish.

▲ - Microsomal preparation from $25^{o}C$ acclimated crayfish.

Figure 6-7b The effect of temperature upon the pH of the medium used for assay of 'Basal' Mg^{2+}- dependent ATPase activity.

pH measurement: The pH was measured on a Beckman 'Expandomatic' pH Meter at different temperatures

Medium: The medium contained 6mM $MgCl_2$, 1.0 mM EGTA, 200mM KCl, 50mM Imidazole - HCl PH7.1 in deionised water.

Legend: Ordinate - pH

Abscissa - Temperature (^{o}C)

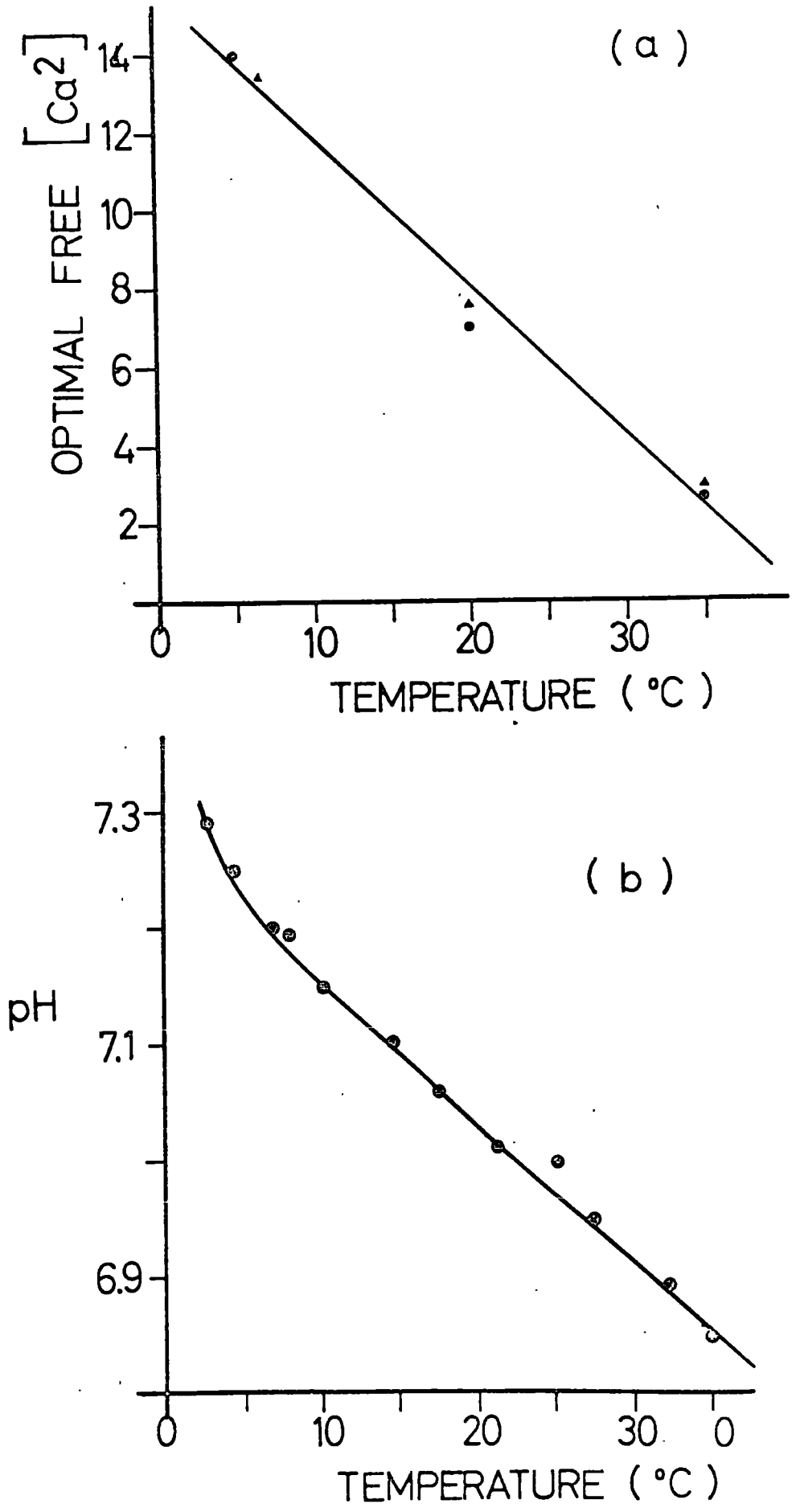

(a)
OPTIMAL FREE [Ca2]
14
12
10
8
6
4
2
0
10
20
30
TEMPERATURE (°C)
(b)
pH
7.3
7.1
6.9
0
10
20
30
0
TEMPERATURE (°C)

Figure 6-8: The time-course of ATP hydrolysis, in the presence of 0.1mM ATP, by a microsomal Ca^{2+}-stimulated ATPase isolated from 4°C acclimated crayfish.

Assay Conditions: 'Total' Ca^{2+}-Mg^{2+}-ATPase activity was determined in the presence of 0.5mM $CaCl_2$, 0.5mM EGTA, 3mM $MgCl_2$, 75mM Sucrose, 100mM KCl, 25mM Imidazole -HCl pH 7.1 and 0.1mM ATP. 'Basal' Mg^{2+} ATPase was assayed in the same medium but omitting $CaCl_2$. Values plotted (Ca^{2+}-stimulated ATPase) were the difference between 'Total' and 'Basal' ATP hydrolysis for each incubation period.
For other details see 'Materials and Methods'.

Legend: Ordinate - ATP hydrolysis (uM Pi liberated. mg protein^{-1}.)
Abscissa - Period of Incubation (minutes)

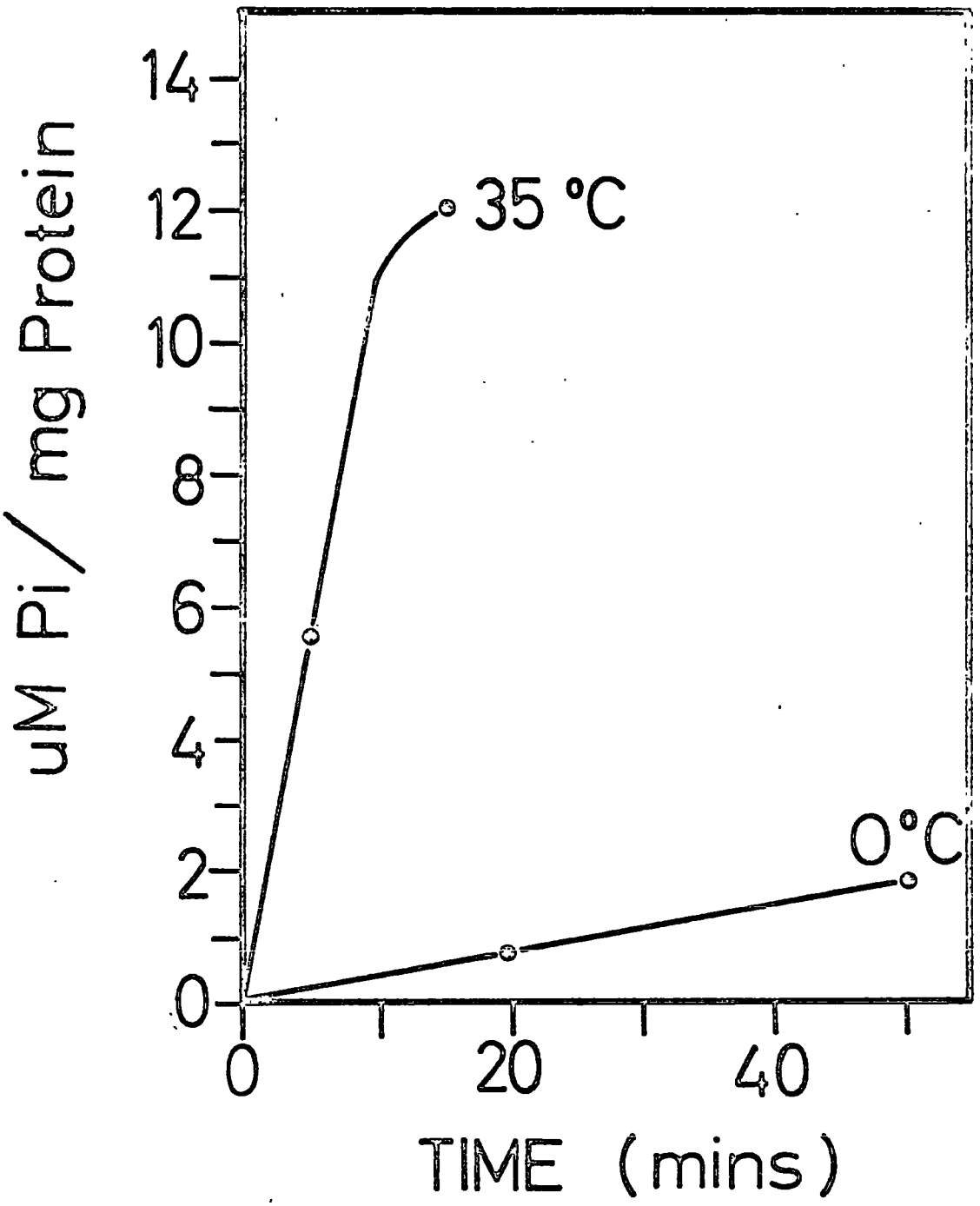
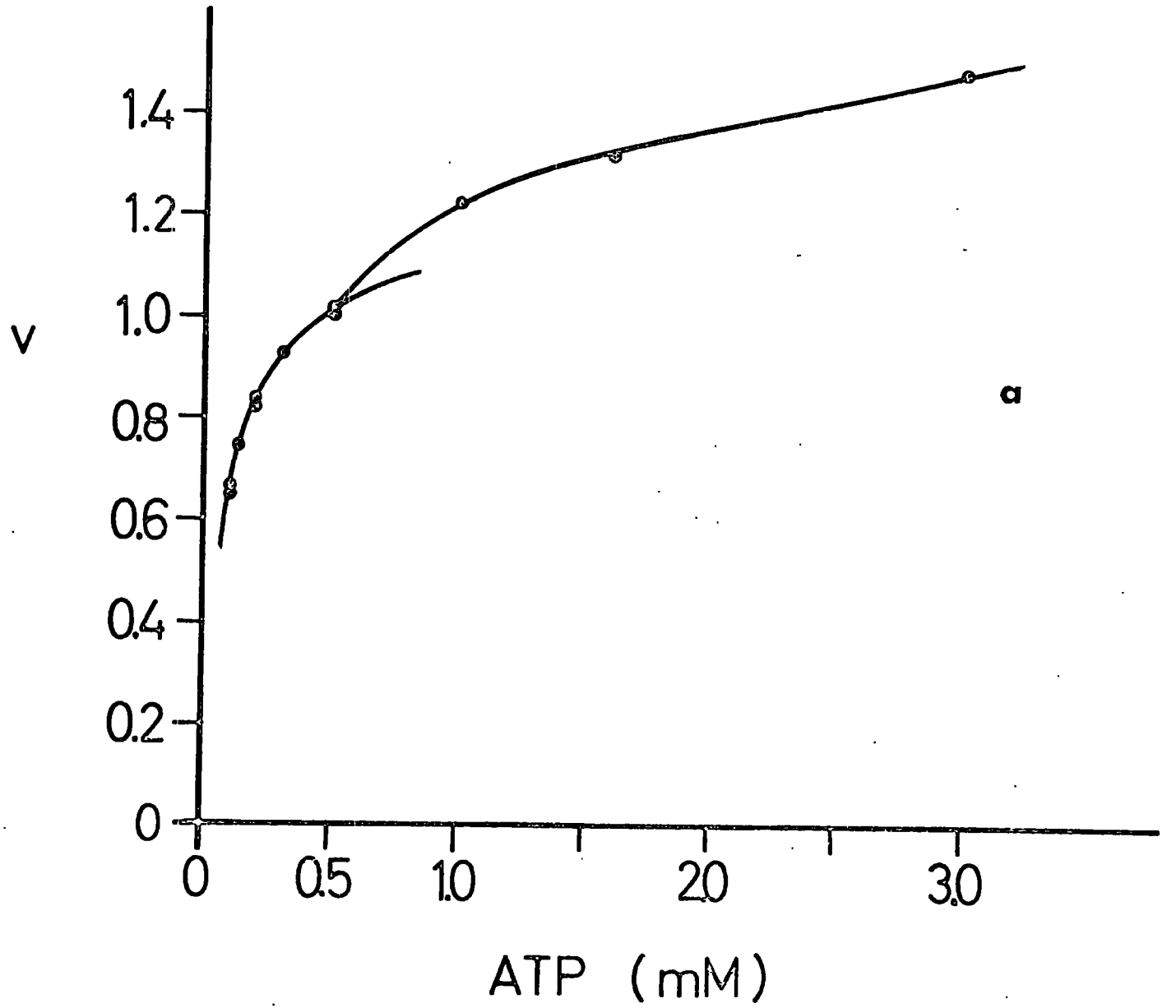

uM Pi / mg Protein
14
12
10
8
6
4
2
0
35 °C
0°C
0
20
40
TIME (mins)

Figure 6-9(a): The ATP saturation curve at 4°C for a microsomal Ca^{2+}-stimulated ATPase preparation isolated from 4°C acclimated crayfish, together with (b) the corresponding Lineweaver-Burk plot.

Assay Conditions: 'Total' Ca^{2+}, Mg^{2+}, ATPase activity was assayed in the presence of 0.5 mM $CaCl_2$, 0.5 mM EGTA, 3 mM $MgCl_2$, 75 mM Sucrose, 100 mM KCl, 25 mM Imidazole-HCl, pH 7.1 and 0.1-3.0 mM ATP. 'Basal' Mg^{2+} ATPase was assayed in the same media but omitting $CaCl_2$. Values plotted (Ca^{2+}-stimulated ATPase) are the difference between the 'total' ATPase and the 'basal' ATPase activity at each ATP concentration.

Legend:
(a) Ordinate: Ca^{2+}-stimulated ATPase activity (μM Pi. mg protein^{-1}. minute^{-1}.)
Abscissa: ATP concentration (mM)
(b) Ordinate: $1/Ca^{2+}$ ATPase activity (μM Pi. mg protein^{-1}. minute^{-1}.)$^{-1}$
Abscissa: 1/ATP (mM^{-1})


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
V
0
0.5
1.0
2.0
3.0
ATP (mM)
a

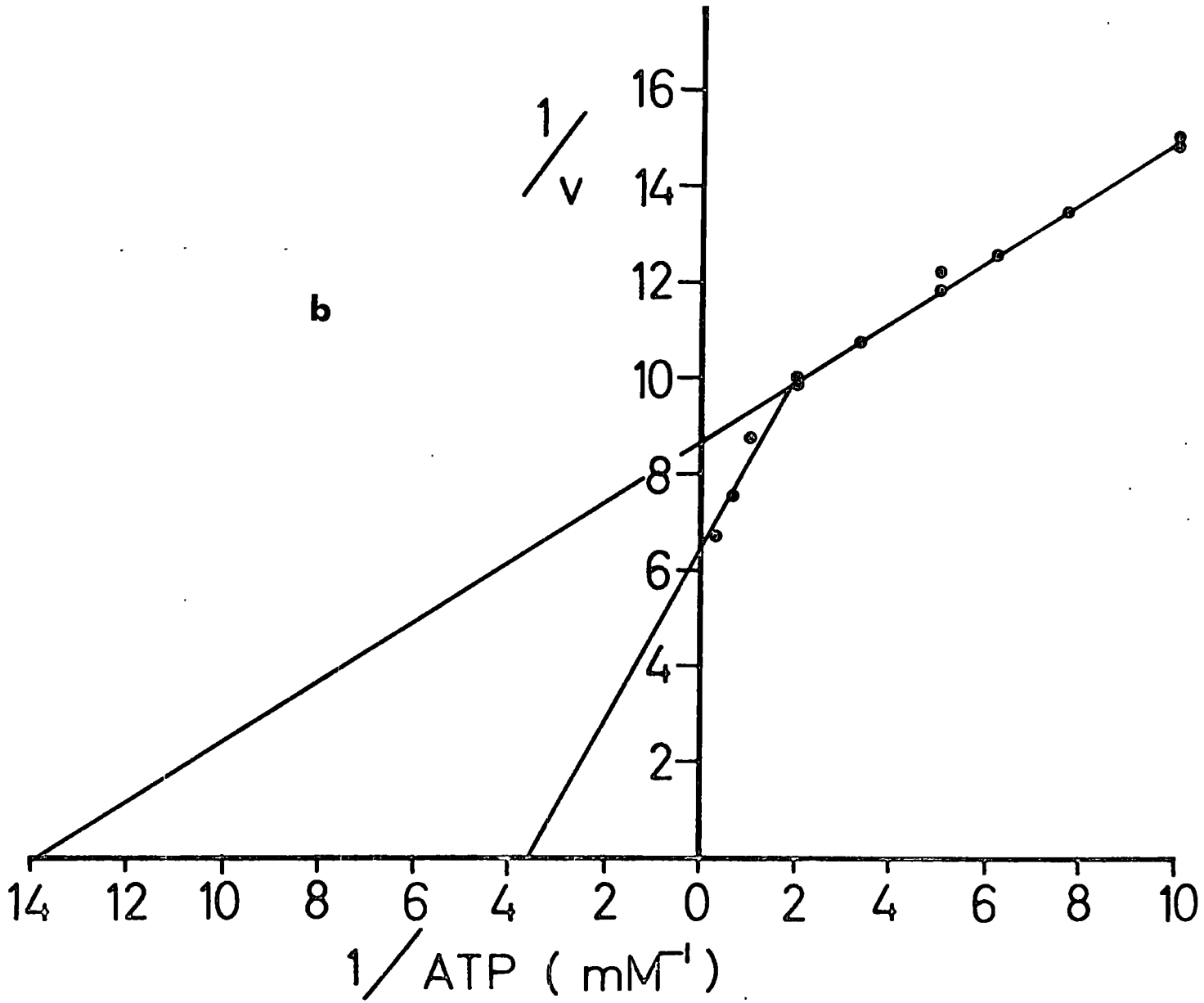

b
1/v
16
14
12
10
8
6
4
2
14 12 10 8 6 4 2 0 2 4 6 8 10
1/ATP (mM⁻¹)

Figure 6-10: The ATP saturation curves at different incubation temperatures for a single microsomal Ca^{2+}-stimulated ATPase preparation isolated from $4^{o}C$ acclimated crayfish.

Assay Conditions: The 'Total' Ca^{2+}-Mg^{2+}-ATPase was assayed in the presence of 0.5mM $CaCl_2$, 0.5mM EGTA, 3mM $MgCl_2$, 100mM KCl, 75mM Sucrose, 25mM Imidazole -HCl pH 7.1 and 0.1-3.0mM ATP. 'Basal' Mg^{2+}-dependent, ATPase was assayed in the same medium, omitting $CaCl_2$.

Values plotted are the difference between the 'Total' ATPase and the 'Basal' ATPase activity at each ATP concentration. The original data is presented in Table 6-4. For other details see 'Materials and Methods'.

Legend: Ordinate - Ca^{2+}-stimulated ATPase activity (uM Pi liberated. mg protein^{-1}. minute^{-1}.)

Abscissa - Concentration of ATP (mM)

● - $0^{o}C$ Incubation Temperature

○ - $4^{o}C$ " "

▲ - $8^{o}C$ " "

△ - $12^{o}C$ " "

◇ - $18^{o}C$ " "

☆ - $23^{o}C$ " "

□ - $28^{o}C$ " "

○ - $35^{o}C$ " "

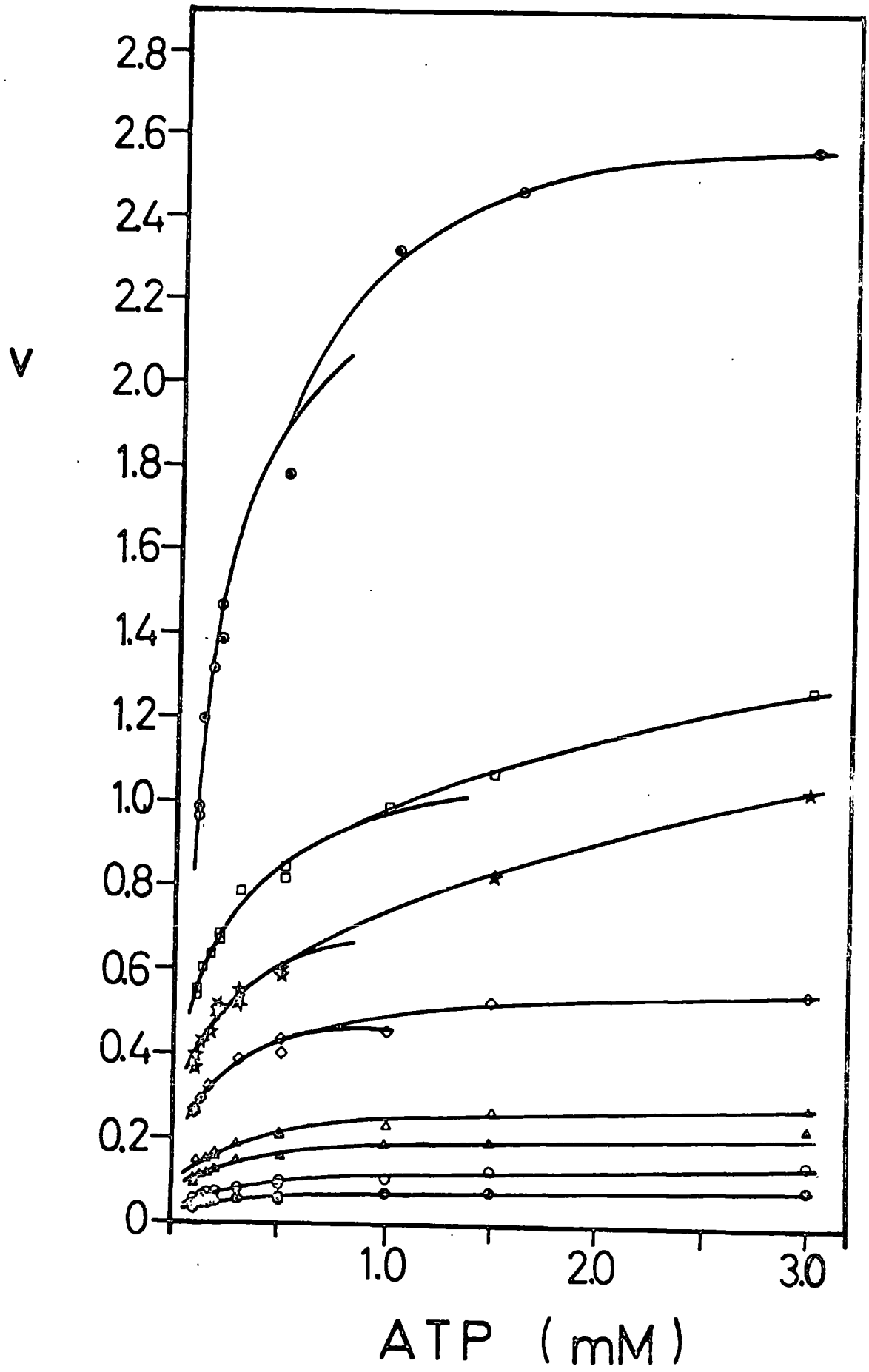

V
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1.0
2.0
3.0
ATP (mM)

Figure 6-11: The ATP saturation curves at different incubation temperatures for a single microsomal Ca^{2+}-stimulated ATPase preparation isolated from 25°C acclimated crayfish.

Assay Conditions: The 'Total' Ca^{2+}-Mg^{2+}-ATPase was assayed in the presence of 0.5mM $CaCl_2$, 0.5mM EGTA, 3mM $MgCl_2$, 75mM Sucrose, 100mM KCl, 25mM Imidazole -HCl pH7.1 and 0.13.0mM ATP. 'Basal' Mg^{2+}-dependent ATPase was assayed in the same medium, omitting $CaCl_2$. Values plotted are the difference between the 'Total ATPase and the 'Basal' ATPase and the 'Basal' ATPase activity at each ATP concentration. The original data is presented in Table 6-5. For other details see 'Materials and Methods'.

Legend: Ordinate - Ca^{2+}-stimulated ATPase activity (uM Pi liberated. mg protein^{-1}. minute^{-1}.)

Abscissa - Concentration of ATP (mM)

- ● - 0°C Incubation Temperature
- ○ - 4°C " "
- ▲ - 8°C " "
- △ - 12°C " "
- ◇ - 18°C " "
- ☆ - 23°C " "
- □ - 28°C " "
- ○ - 35°C " "

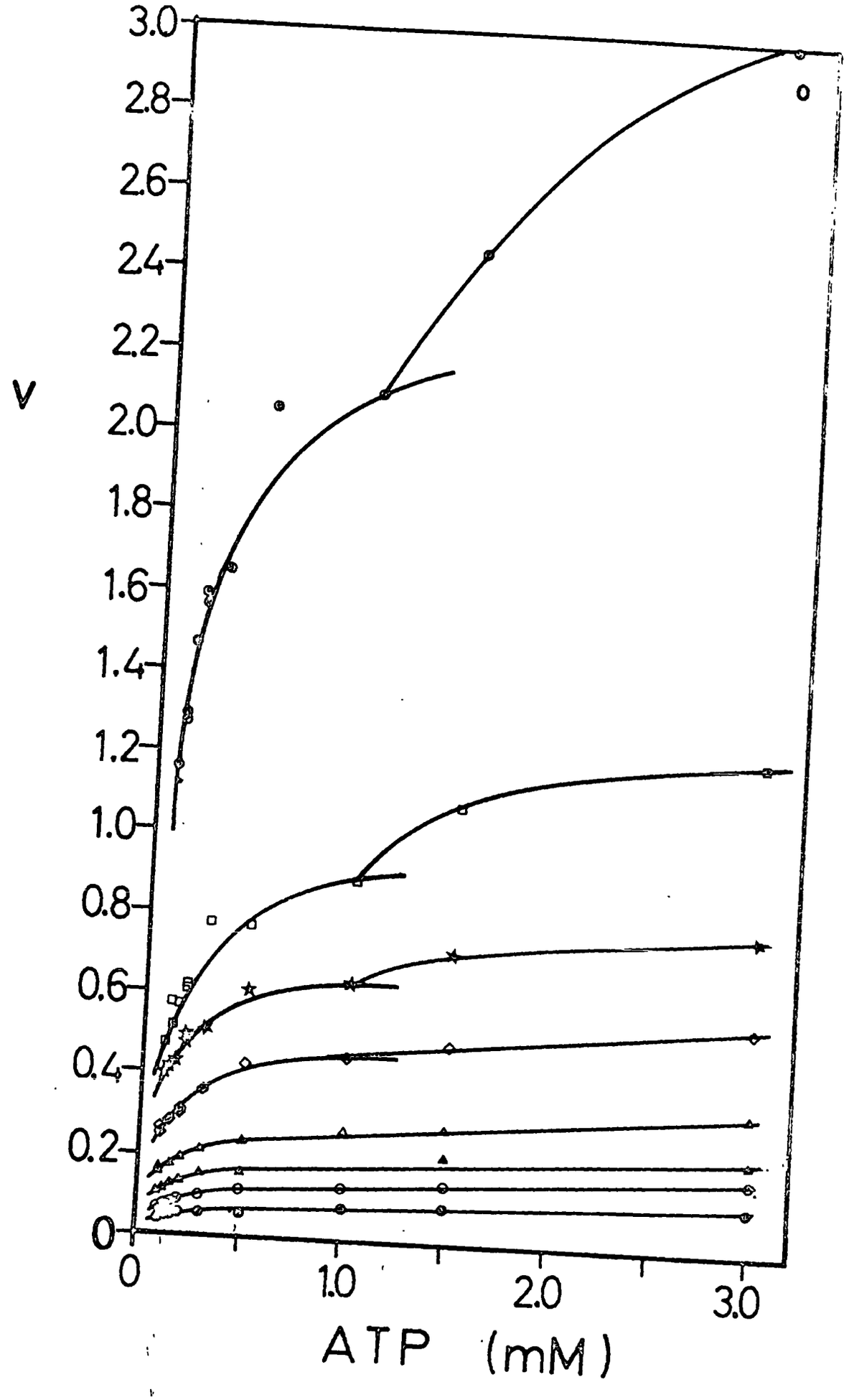

3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
V
0
1.0
2.0
3.0
ATP (mM)

Figure 6-12: The Lineweaver-Burk plots of the saturation with ATP at different temperatures, of the microsomal Ca^{2+}-stimulated ATPase isolated from the muscle of $4^{o}C$ acclimated crayfish

Assay Conditions: The Ca^{2+}-stimulated ATPase was determined at several ATP concentrations between 0.1 - 3.0mM ATP, at each incubation temperature. Details of the assay procedure may be found in 'Materials and Methods' and Figure 6-10.

Legend: Ordinate - $\frac{1}{v}$ (uM Pi liberated. mg protein^{-1}. minute^{-1}.)$^{-1}$

Abscissa - $\frac{1}{ATP}$ $(mM)^{-1}$

■ - - $0^{o}C$ Incubation temperature

⊙ - $4^{o}C$ " "

□ - $8^{o}C$ " "

▲ - $12^{o}C$ " "

○ - $18^{o}C$ " "

◆ - $23^{o}C$ " "

△ - $28^{o}C$ " "

◇ - $35^{o}C$ " "

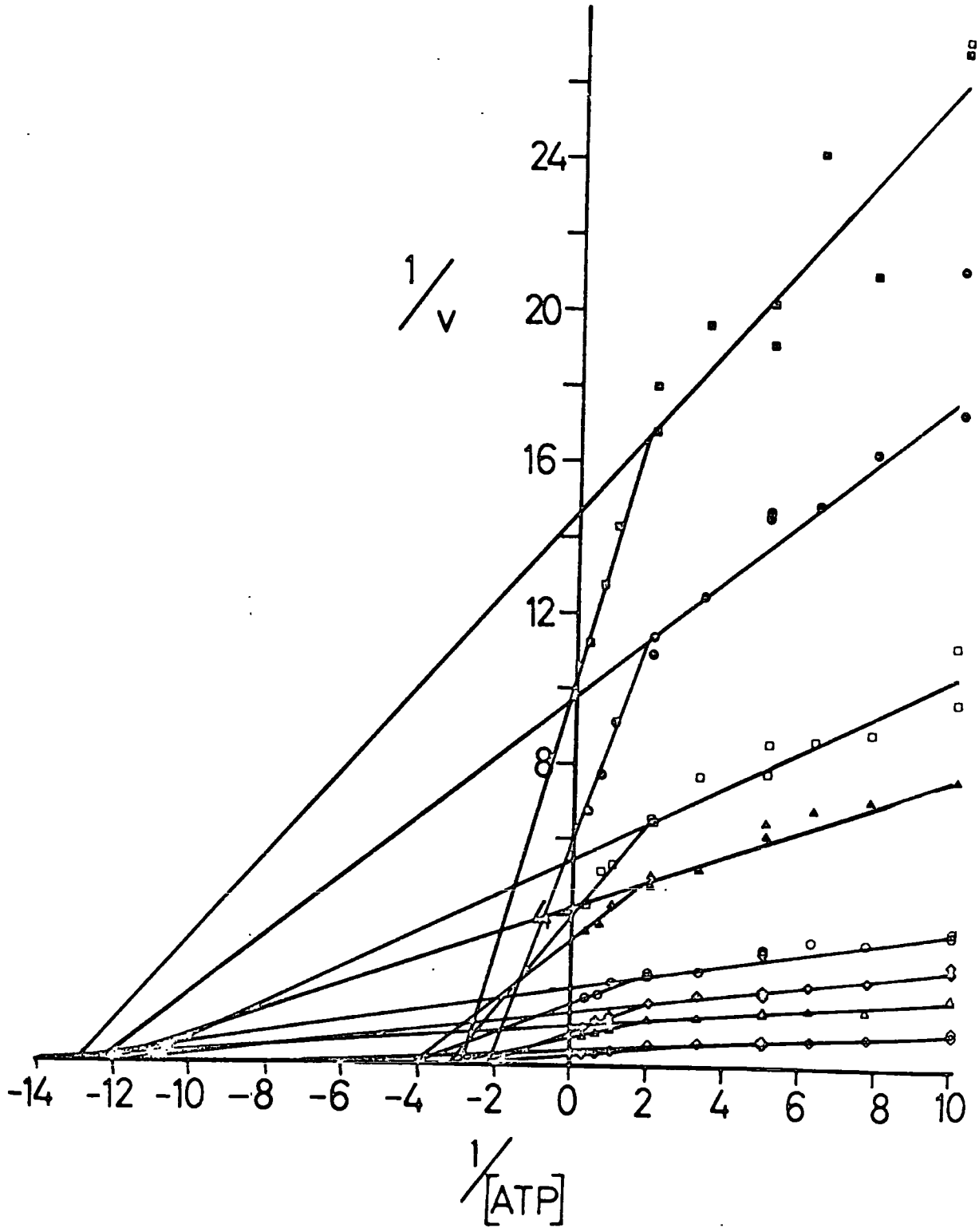

1/v
24
20
16
12
8
-14
-12
-10
-8
-6
-4
-2
0
2
4
6
8
10
1/[ATP]

Figure 6-13: The Lineweaver - Burk plots of the saturation with ATP at different temperatures, of the microsomal Ca^{2+}- stimulated ATPase isolated from the muscle of 25°C acclimated crayfish.

Assay Conditions: The Ca^{2+}- stimulated ATPase was determined at several ATP concentrations between 0.1-3.0mM ATP, at each incubation temperature. Details of the assay procedure may be found in 'Materials and Methods', and Figure 6-11.

Legend: Ordinate - $^{1}/_{v}$ (uM Pi liberated. mg protein^{-1}. minute^{-1}.)$^{-1}$

Abscissa - $^{1}/_{[ATP]}$ (mM)$^{-1}$

■ - 0°C Incubation Temperature

● - 4°C " "

□ - 8°C " "

▲ - 12°C " "

○ - 18°C " "

◆ - 23°C " "

△ - 28°C " "

◇ - 35°C " "

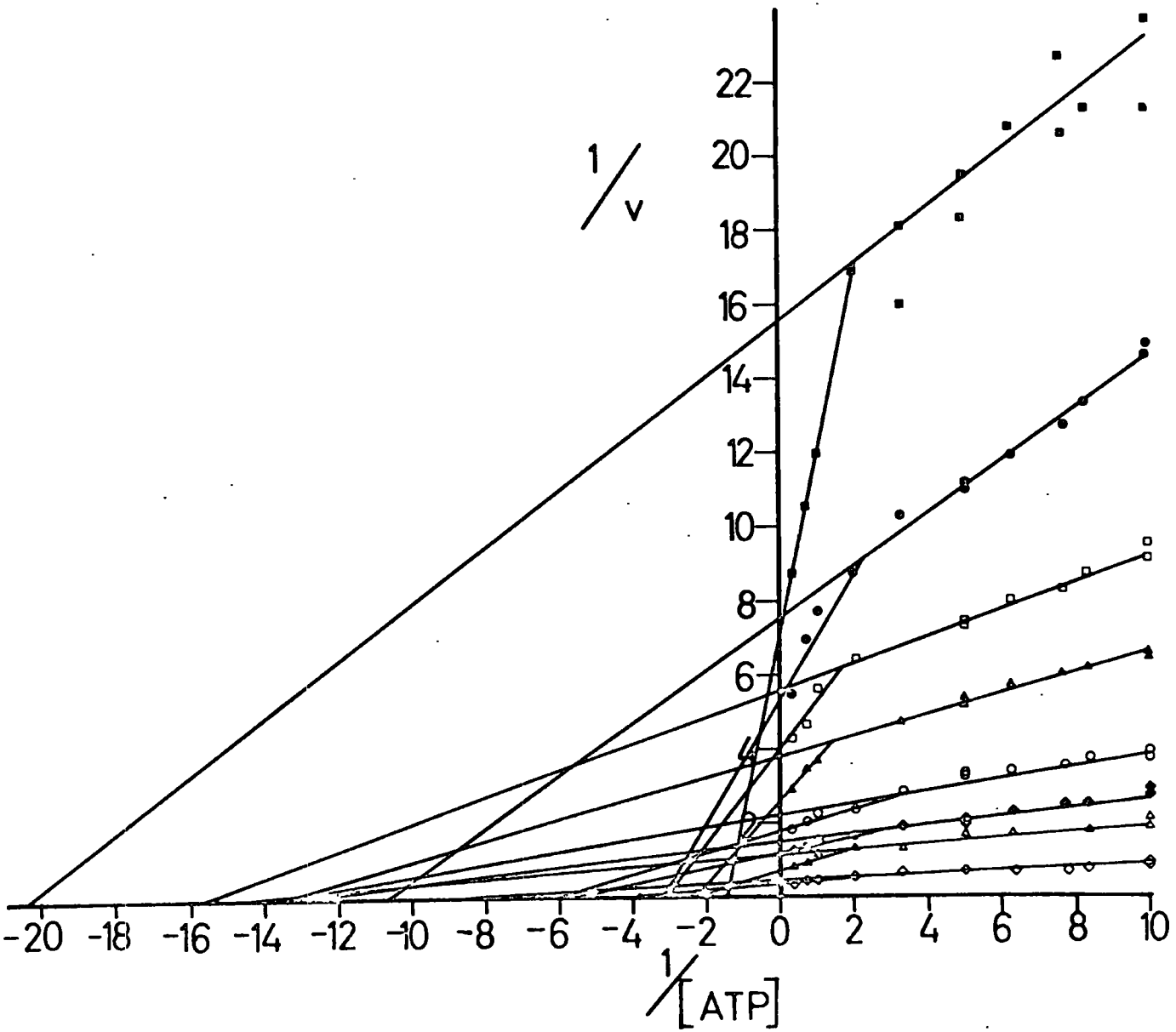

1/v
22
20
18
16
14
12
10
8
6
-20 -18 -16 -14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10
1/[ATP]

Figure 6-14: The effect of temperature upon the Km (ATP) at low ATP concentrations of the microsomal Ca^{2+}- stimulated ATPase isolated from the muscle of (a) 4°C and (b) 25°C acclimated crayfish.

Assay Conditions: For details, see Table 6-7 and 6-9 for the preparations isolated from the muscle of 4°C and 25°C acclimated crayfish respectively, and 'Materials and Methods'.

Legend: Ordinate : Km for ATP (mM).

Abscissa : Incubation Temperature (°C).

Km ± S.E., calculated from regression analysis of Lineweaver - Burk plot.

N.B. The standard error above and below Km are not equal. See 'Materials and Methods'.

Km ± S.E., calculated from regression analysis of Eadie - Hofstee plot.

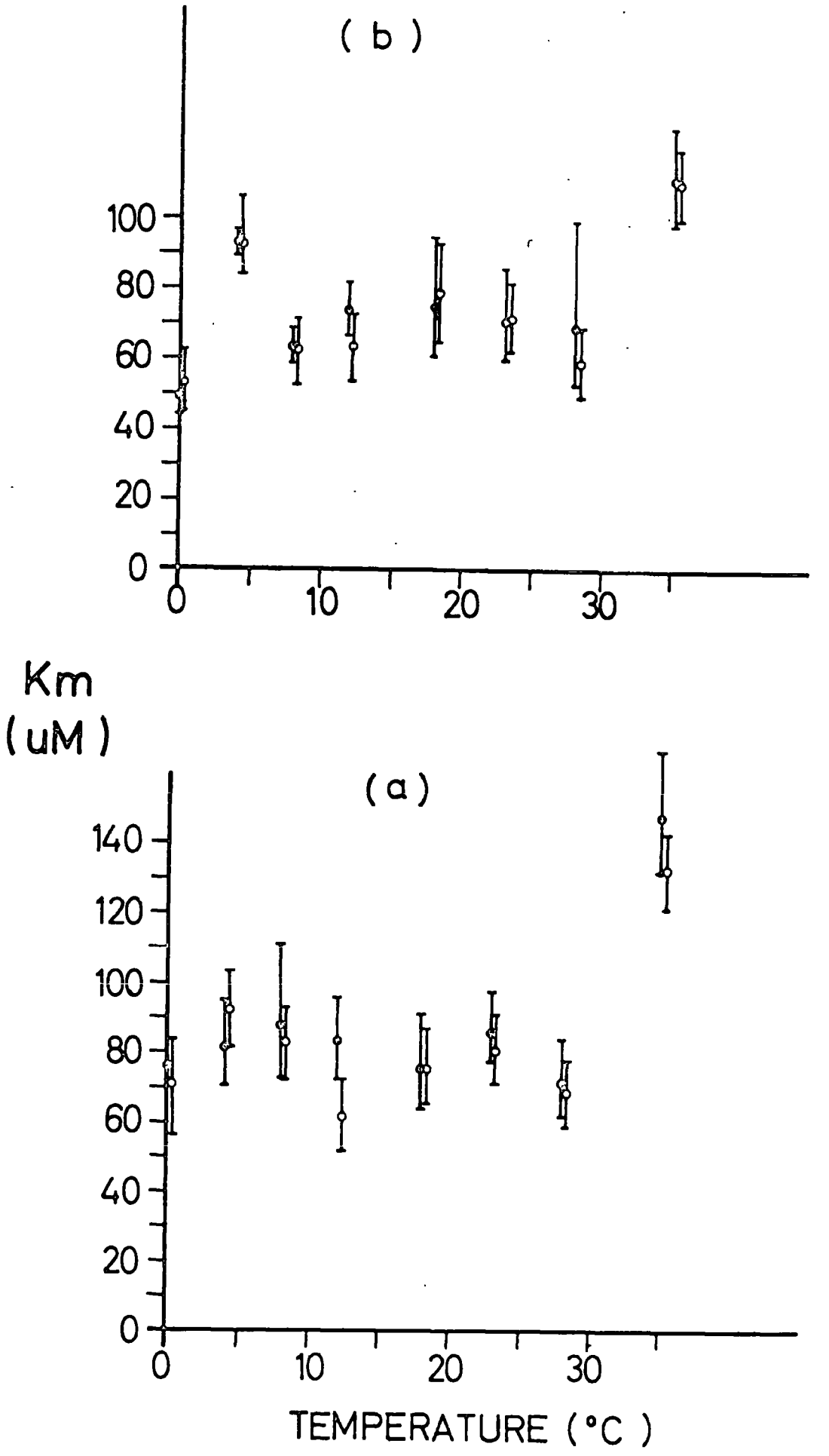

(b)
100
80
60
40
20
0
0
10
20
30
Km
(uM)
(a)
140
120
100
80
60
40
20
0
0
10
20
30
TEMPERATURE (°C)

Figure 6-15: The effect of temperature upon the Km (ATP) at high ATP concentrations of the microsomal Ca^{2+}- stimulated ATPase isolated from the muscle of (a) $4^{o}C$ and (b) $25^{o}C$ acclimated crayfish.

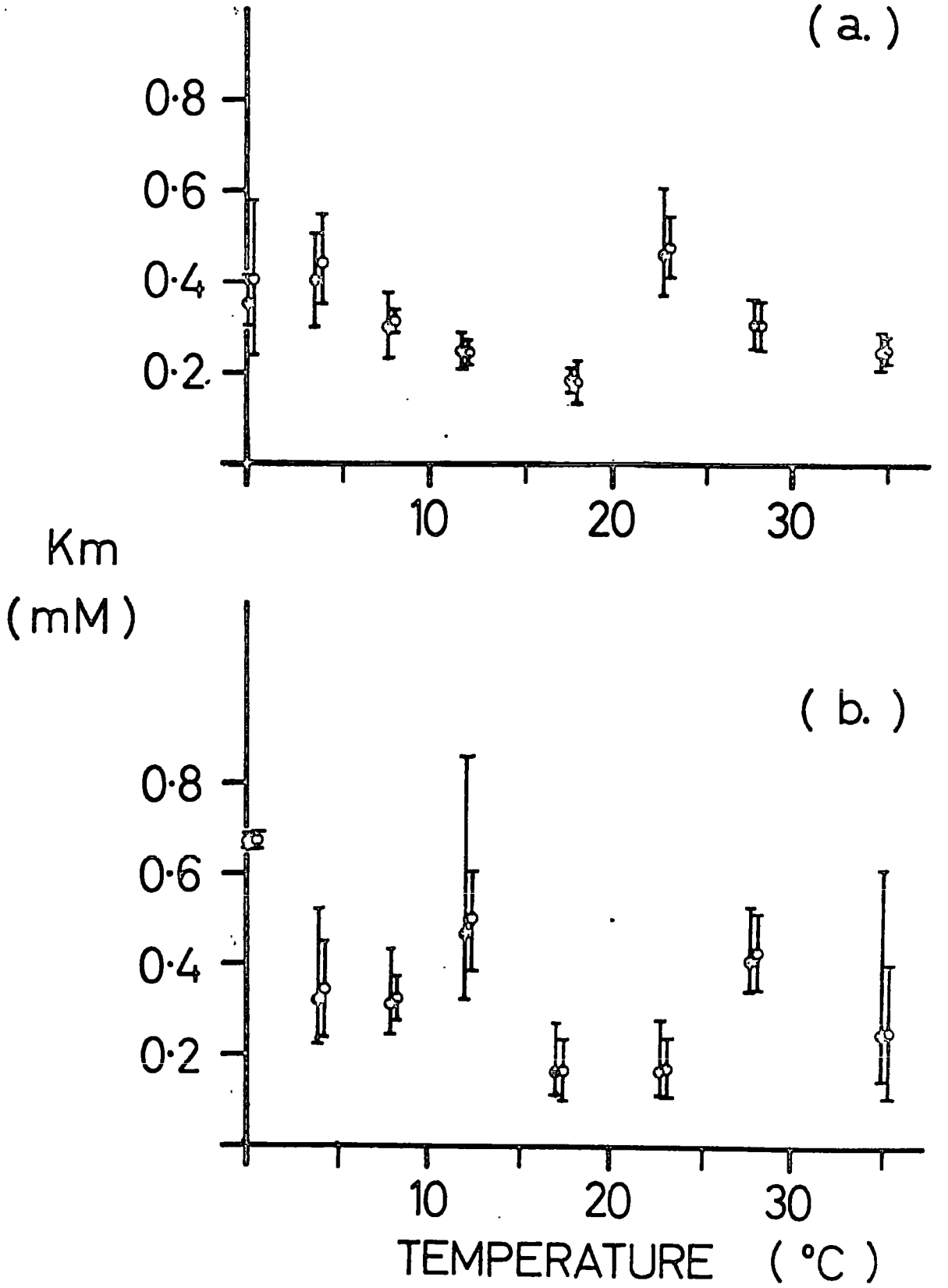
Assay Conditions: For details, see Table 6-6 and 6-8 for the preparations isolated from the muscle of $4^{o}C$ and $25^{o}C$ acclimated crayfish respectively, and 'Materials and Methods'.

Legend: Ordinate : Km for ATP (mM)

Abscissa : Incubation Temperature (^{o}C)

Km $\pm$S.E., calculated from regression analysis of Lineweaver - Burk plot. N.B. The standard error above and below Km are not equal. See 'Materials and Methods'.

Km $\pm$ S.E., calculated from regression analysis of Eadie - Hofstee plot.


(a.)
0·8
0·6
0·4
0·2
10
20
30
Km
(mM)
(b.)
0·8
0·6
0·4
0·2
10
20
30
TEMPERATURE (°C)

Chapter 7

THE EFFECT OF TEMPERATURE AND THERMAL ACCLIMATION UPON ENZYME ACTIVITY

INTRODUCTION

The temperature characteristics of membrane-bound enzymes and multienzyme systems are often complicated by the presence of discontinuities in their Arrhenius plots, as has been demonstrated in certain respiratory enzymes of mitochondria (Kemp, Groot and Rietsma,1969; Lyons and Raison, 1970; Raison, Lyons and Thomson,1971; Lenas, Sechi, Parenti-Castelli, Landi and Bertoli 1972; Smith,1973; for review see Raison,1972), membrane-bound ribosomal protein synthesis (Towers, Raison, Kellerman and Linnane,1972), the Na^+-K^+-ATPase (Gruener and Avi-Dor,1966; Bowler and Duncan 1968; Charnock, Cook and Casey,1971; Taniguchi and Iida,1972; Tanaka and Teruya,1973) and the Ca^{2+}-Mg^{2+}-ATPase of the sarcoplasmic reticulum (Charnock and Frankel,1971; Deamer,1973). They usually consist of two straight lines, of different slopes, which intersect at a characteristic temperature of transition, such that above this temperature the process has an activation energy (Ea) of approximately 5-15 Kcals $mole^{-1}$ (20.9 - 62.8 KJ. $mole^{-1}$.), whilst below this temperature it becomes 20-30 Kcals $mole^{-1}$ (83.7 - 125.6 KJ. $mole^{-1}$.).

Crozier and his co-workers (quoted by Johnson, Eyring and Polissar 1954), from certain physiological studies, have suggested that this change in activation energy represents a shift from one rate limiting reaction to another. Belehradek (1935, 1957) and Dixon and Webb (1964) have argued that two simultaneously occurring processes of this type would not

yield a sharp inflexion of an Arrhenius plot but would produce a smooth curvilinear transition. The sharp discontinuity was considered to be the result of incorrectly drawing the intercept of tangents to smooth curves.

The interpretation of discontinuities of Arrhenius plots in enzyme studies has been further complicated by recent data (Lyons and Raison 1970a, b), which yield Arrhenius plots consisting of two straight lines of differing slopes, which do not intercept at the temperature of transition. Kumamoto, Raison and Lyons (1971) considered these sharp breaks were real. They further suggested that the breaks can only be the result of a phase change within the system at the transitional temperature. Each process is thought to function independently over adjacent temperature ranges and therefore they do not occur simultaneously. This effectively negates the theoretical arguments for a curvilinear Arrhenius plot as proposed by Belehradek (1935, 1957). The phase change was thought to cause some conformational change in the enzyme, its substrate or its solvent environment, which produces a process with distinctly different activation energies above and below the transitional temperature. This analysis supports the notion that intersecting breaks in Arrhenius plots are real and not the result of incorrect graphical procedures.

There is growing evidence that in membrane-bound enzymes conformational changes can be the result of a phase change in the non-polar hydrocarbon environment of the enzyme, changing from a rigid, crystalline lattice at temperatures below the transition temperature, to a more fluid, liquid-crystalline state above the transition temperature. For example, several workers have established a close correlation between the transition temperature of enzyme activity and the

transition temperature of the molecular mobility parameter of a hydrophobic probe, as detected by Electron Paramagnetic Resonance Spectroscopy (Raison, Lyons, Melhorn and Keith,1971; Grisham and Barnett,1973; Inesi and Eletr,1972; Inesi, Millman and Eletr,1973) or Fluorescence Spectroscopy (Rottem, Cirillo, De Kruyff, Shinitsky and Razin,1973). In addition, Grisham and Barnett (1973) have demonstrated that the molecular mobility of a hydrophobic probe in the extracted lipids from a Na^+-K^+-ATPase preparation has an identical transition temperature to the molecular mobility of the same probe in the in vitro membrane preparation and also the ATPase activity.

Furthermore, delipidated membrane enzymes can be reactivated by added phospholipids. It is significant that the temperature characteristics of reconstituted enzymes depend solely upon the nature of the added lipids and not upon the enzymes per se (Seelig and Hasselbach,1971; Tanaka and Teruya,1973; Kimelberg and Papahadjopoulos,1974). These observations on well-defined, reconstituted systems were given further credance by the work of Wilson, Rose and Fox (1970). They found that the temperature characteristics of in vivo transport activity of the unsaturated fatty acid auxotrophs of Escherischia coli K12 were influenced mainly by the fatty acid composition of the membranes. This was determined primarily by the nature of the exogenous fatty acid supply. Rottem, Cirillo, De Kruyff, Shinitsky and Razin (1973) have demonstrated that in Mycoplasma mycoides, the transition temperature of the plasma membrane ATPase activity depended upon the fatty acid composition of the membrane lipids. Janki, Aithal, McMurray and Tustanoff (1974) have induced similar changes in both catalytic and Arrhenius kinetics of mitochondrial enzyme characteristics of both inner and outer mitochondrial membranes by growing the

yeast Saccharomyces cerevisiae in a minimal medium supplemented with certain fatty acids.

Thermally-induced phase transition in mitochondrial membranes and changes in the Ea of their enzymes are commonly observed (Lyons and Raison,1970), with membranes from homoeotherms and chill-sensitive plants. However, they are not observed often in similar preparations from poikilotherms (Lyons and Raison,1970a; Smith,1973) and chill-resistant plants (Lyons and Raison,1970b; Raison, Lyons and Thomson, 1971; Feeney, Vandenheede and Osaga,1972). This is correlated with a higher proportion of polyunsaturated fatty acids, and an increased membrane fluidity. Cold adapted organisms usually have a membranous phase transition at temperatures below $0^{o}C$.

Finally, the discontinuity in Arrhenius plots could be abolished by treatments that affected the physical properties of the membrane lipids. Thus the presence of detergents and preincubation with phospholipases was found to induce linear Arrhenius plots (Taniguichi and Iida,1972; Raison, Lyons, Thomson,1971; Charnock, Cook and Casey,1971). The discontinuity is not affected by a period of storage or by the specific activity of the preparation and is not related to the specific activity of the preparation. However, caution is urged in this respect since some workers have found that phospholipase A (Charnock, Cook, Almeida and To, 1973) and various detergents (Charnock, Cook, Almeida and To,1973; Smith,1973) had no effect on the breaks in Arrhenius plots of certain mitochondrial enzymes.

There is now no doubt that such breaks are not artefacts and that these temperature-induced changes in Arrhenius plots of enzyme activity are probably a consequence of a phase

change in the lipid component of the membrane, rather than an intrinsic property of the enzyme. However, it should be pointed out that the nature of the role of lipid phase changes in regulating membrane enzymes is not well understood, but is probably related to the conformational changes discussed by Tasaki (1968) and observed by Papakostidis, Zundel and Mehl (1972). It is thought that lipids in the crystalline state restrict the conformational flexibility of the enzyme and increase the ΔH^* required for its activation. Inesi, Millman and Eletr (1973) have measured the ΔS^* for ATPase activity and Ca^{2+}-uptake by rabbit sarcoplasmic reticulum. Negligible quantities for entropy of activation were obtained for both functions at temperatures above the break, whereas large positive values of ΔS^* were observed at temperatures below the break. They concluded that the highly ordered lipid environment at low temperatures "impedes their translational and/or rotational freedom" (Inesi, Millman and Eletr 1973).

The phase transition in membranes is now recognised to be a more complex and co-operative event than originally envisaged. Membranes with a complex phospholipid composition and diverse fatty acyl constituents have been found to have a transition temperature that occurs over a broad temperature range of approximately 10-20^{o}C, as judged by Differential Scanning Calorimetry (Chapman, Urbina and Keough,1974). Hence at any one temperature in the transitional zone, there may be phospholipids in both the crystalline and liquid-crystalline states, resulting in a heterogenous membrane structure. It has been demonstrated that the particles revealed by Freeze-Etch Microscopy preferentially aggregate in the fluid region of the membrane (James, Branton, Wisnieski,

Keith 1972; Speth and Wunderlich,1973; Kleeman and McConnell, 1974). If these particles are indeed hydrophobic proteins, they would appear to enter the crystalline environment only when all the membrane lipid has become crystalline. This phenomenon would account for the relatively sudden transition observed in the Arrhenius plots of membrane-bound enzymes and hydrophobic probes, that correspond to the start of the lipid-melt observed by Differential Sacnning Calorimetry (Kimelberg and Papahadjopoulos,1974; Inesi, Millman and Eletr,1973; De Kruyff, Van Dijck, Goldback, Demel and Van Deenen,1973). In addition, the transition is influenced not only by the types of phospholipids present, the chain length, degree and type of unsaturation of their constituent fatty acids but also by the pH, ionic content (Trauble and Eibl,1974) and divalent-cation content of the aqueous environment (Chapman, Urbina and Keough,1974; Verkleij, De Kruyff, Ververgaert, Tocanne and Van Deenen,1974). Cholesterol has the effect of removing these membrane phase transitions when present at sufficiently high concentrations (Ladbrooke, Williams and Chapman,1968).

In view of these complications, it is difficult to determine the in vivo significance of transition phenomena and whether they play an important role in cellular metabolism of poikilotherms. Eletr, Williams, Watkins and Keith (1974) have shown that in a yeast culture at least, perturbation of lipid interactions in membrane systems as detected by the motion of spin labels, results in a similar perturbation of a physiological activity of the whole cell (i.e., oxygen uptake). They have also demonstrated tnat the transition temperatures of both phenonema are sensitive to the membrane fatty acid composition as influenced by the exogenous fatty acid supply.

In conclusion, these experiments indicate that enzyme activity and its temperature characteristics can, with certain reservations, be extremely useful "probes" of their lipid environment. The experiments described in this chapter were designed to answer the following questions concerning the temperature characteristics of the microsomal Ca^{2+}-Mg^{2+}-ATPase of crayfish abdominal muscle. Firstly, is the Ca^{2+}-stimulated ATPase activity of the microsomal preparation affected by the acclimatory history of the animal? Secondly, does the Arrhenius plot of enzyme activity (Vmax, or v) show any dependence upon the acclimation history of the crayfish, which may be correlated with the accompanying changes in membrane fatty acid composition (Chapter 3)? Thirdly, does enzyme activity show a reduced dependence upon temperature at low substrate concentrations as has been observed in several soluble enzymes (see Hochachka and Somero 1973)? Fourthly, does the activation energy of the Ca^{2+}-stimulated ATPase change during temperature adaptation? This might indicate a change in the enzyme machinery caused either by a change in the type of isoenzyme present or by modification of the existing enzyme. Fifthly, does the Ca^{2+}-stimulated ATPase show any evolutionary adaptation towards continued efficient function in a cold environment, such as a reduction of activation energy when compared to homoeotherms, or warm-adapted poikilotherms?

MATERIALS AND METHODS

A. MATERIALS

a. Animals: Adult *Austropotamobius pallipes* were caught and maintained in the laboratory as described in Chapter 2.

b. Chemicals: See Chapter 5.

c. Glassware: The glassware used has been described in Chapter 5.

B. METHODS

a. Preparation of microsomes. The 13,000g - 35,000g heavy microsomal fraction was obtained from crayfish muscle homogenates as described in Chapter 5. The final pellet was resuspended in 0.3M sucrose in 10mM Imidazole -HCl pH 7.1.

b. Compositions of assay media. These are described in Chapter 5.

c. Incubation procedure. Temperature gradients were set up using a thick aluminium bar (1.20 x 0.10 x 0.06m) with a series of holes drilled at short intervals along its length, to accommodate the assay tubes. A crushed ice bath at one end and a hot water bath at the other provided a gradient of temperatures between 0°C and 40°C. 1ml of an ionic medium was added to each tube followed by 0.5ml ATP (12mM concentration). After a 10 minute thermoequilibration period the reaction was started by adding 0.5ml of the enzyme suspension. The reaction was stopped by adding 4mls of the 'Lubrol' or 'Cirrasol' mixture as described in Chapter 5.

d. Estimation of Enzyme Activity. The amount of inorganic phosphate liberated was analysed by the method of Atkinson, Gatensby and Lowe (1971) as described in Chapter 5.

e. Estimation of Protein Content. Diluted aliquots of the microsomal suspension were analysed for protein by the method of Lowry, Rosebrough, Farr and Randall (1951) as described previously (Chapter 5).

f. Statistical Techniques.

(i) Statistical comparison of data. All statistical comparisons were performed using conventional techniques as described by Snedecor and Cochran (1967). Where appropriate, reference was made to the statistical tables of Fisher and Yates (1963).

(ii) Estimation of Arrhenius activity energy. Arrhenius plots of logv against $\frac{1}{T^{o}K}$ were prepared and the slope (A) of the equation

$$\log v = -\frac{A}{T} + B$$

was determined by regression analysis (Snedecor and Cochran 1967). The Arrhenius activation energy (Ea) was computed from the equation

$$Ea = R \times 2.303 \times A \text{ Kcal. mole}^{-1}$$

Where R - gas constant = 1.987 cal/mole/^{o}K. (8.33 J/mole/^{o}K)

RESULTS

A. The Effect of Thermal Acclimation upon Microsomal ATPase Activity

The specific activity of the Ca^{2+}-stimulated ATPase of several microsomal preparations isolated from 4°C and 25°C acclimated crayfish was measured at 25°C in the presence of 8.1uM free calcium, 3.0mM $MgCl_2$ and 3.0mM Tris-ATP (all final concentrations). Both classes of preparation were found to exhibit quite variable activities, but with average values of 1.311 and 0.988uMoles Pi liberated .mg microsomal protein $^{-1}$.minute^{-1}. for preparations extracted from 4°C and 25°C acclimated crayfish respectively. This does not represent a significant difference. ($P > 0.05$, Table 7-1.)

B. The Effect of Temperature upon Vmax

The data presented in the previous chapter (Tables 6-6 to 6-9) concerning the temperature dependence of Km, permits the effect of temperature upon Vmax to be studied. The Arrhenius plots for Vmax of the Ca^{2+}-stimulated ATPase preparations isolated from 4°C-acclimated crayfish are presented in Figure 7-1 for activity at high and low ranges of ATP concentration, and Figure 7-2 for the preparation isolated from 25°C-acclimated crayfish again at high and low ranges of ATP. In all cases the Arrhenius plots were linear over the temperature range 0°C - 35°C.

The Arrhenius activation energy was calculated by regression analysis of the data (Table 7-2). The activation energy for Vmax at low ATP concen-

trations proved to be somewhat greater than the activation energy for Vmax at high ATP concentrations, in the preparations isolated from both 4^{o}C-acclimated and 25^{o}C-acclimated animals. The activation energy for Vmax at both high and low concentrations of ATP calculated for the preparation isolated from 4^{o}C-acclimated crayfish was higher than the corresponding values for the preparations isolated from 25^{o}C-acclimated crayfish (Table 7-2).

C. The Effect of ATP Concentration upon the Arrhenius Activation Energy

As discussed previously (Chapter 6, Introduction), the temperature dependence of some biochemical processes such as mitochondral oxygen uptake (Newell & Pye 1971) and the activity of some enzymes (see Hochachka and Somero 1973) is dependent upon the substrate concentration. This is thought to lead to a partial or total independence of these processes from the effects of temperature. The experiments reported in the previous chapter enables the effect of substrate concentration upon the activation energy, and also the possibility of temperature-independent enzyme activity to be examined.

The original data is given in Tables 6-4 and 6-5. Figure 7-3 illustrates the dependence upon temperature of the microsomal Ca^{2+}-stimulated ATPase activity of the preparation isolated from 4^{o}C-acclimated crayfish. It is evident from the corresponding Arrhenius plots (Figure 7-5) that the graph at each substrate concentration was

linear over the temperature range 0-35°C. Furthermore, the slope of the lines was not greatly affected by measurement of the reaction velocity at different concentrations of ATP. This conclusion is supported by calculation of the Arrhenius activation energy by regression analysis of the data (Table 7-3). The activation energy, measured at different ATP concentrations, for the preparation isolated from 4°C-acclimated crayfish varied between 15.83 Kcal. $mole^{-1}$ (66.28 KJ. $mole^{-1}$.) and 16.34 Kcals. $mole^{-1}$ (68.41 KJ. $mole^{-1}$.), there being no consistent variation of Ea with ATP concentration.

The preparation isolated from 25°C-acclimated crayfish also exhibited linear Arrhenius plots at all ATP concentrations used (Figures 7-4 and 7-6). The activation energies measured at different ATP concentrations varied between restricted limits of 14.36 Kcal. $mole^{-1}$ (KJ.$mole^{-1}$.) and 15.55 Kcal. $mole^{-1}$ (65.10 KJ. $mole^{-1}$., Table 7-4). This range of Ea values was somewhat lower in the preparation isolated from the 25°C-acclimated crayfish than in the preparation isolated from 4°C-acclimated crayfish. There was no overlap between the two sets of data.

C. Variability between Preparations in Activation Energy of the Microsomal Ca^{2+}-stimulated ATPase

The experiments discussed above were performed on one preparation from each adaptational group. It was considered desirable to know whether the marked differences in the range of activation energies observed with these two preparations

were dependent upon the thermal history of the animal and represented an adaptational difference or whether it was the result of normal variability in the value of the activation energy. This variability may be the result of error in the experimental determination of Ea, or an inherent variability of the preparations.

The Arrhenius plots for a number of different microsomal preparations isolated from 4°C-acclimated crayfish are presented in Figure 7-7 for enzyme activity at 3.0mM ATP, and in Figure 7-8 for activity at 0.5mM ATP. The corresponding Arrhenius plots for microsomal preparations isolated from 25°C-acclimated crayfish are illustrated in Figures 7-9 and 7-10 for activity at 3.0mM ATP and 0.5mM ATP respectively. All Arrhenius plots were linear over the range 0-35°C. The results of regression analysis of these plots and the calculated value of Ea at 0.5mM and 3.0mM ATP, for each experiment are given in Tables 7-5 and 7-6 for preparations isolated from 4°C and Tables 7-5 and 7-7 for preparations isolated from 25°C-acclimated crayfish muscle respectively. These data are summarised in Table 7-8 where the mean activation energy of microsomal preparations isolated from 4°C and 25°C-acclimated crayfish are compared at 0.5mM and 3.0mM ATP. In both cases there was no significant difference between the mean Ea (Table 7-8; $P > 0.8$ for activity at 3.0mM ATP, $P > 0.7$ for activity at 0.5mM ATP). It was therefore concluded that there was no relationship

between the acclimation history of a group of animals and the temperature dependence of a $Ca^{2+}Mg^{2+}$ ATPase preparation derived therefrom.

The results discussed earlier (section B) indicated that the activation energy with respect to Vmax for low [ATP] activity was somewhat greater than the corresponding value for high [ATP] activity. Using the data from Tables 7-5 and 7-6 it was possible to compare the activation energy for enzyme activity at 0.5mM ATP (i.e., low [ATP] activity) and 3.0mM ATP (i.e., high [ATP] activity) for microsomal preparations isolated from 4°C-acclimated crayfish (Table 7-9). The difference was not significant ($P > 0.9$), thus the results obtained in the earlier analysis are presumably caused by normal variability in Ea values and have no adaptive significance.

DISCUSSION

A. The Effect of Temperature Acclimation on Microsomal Enzyme Activity

The microsomal Ca^{2+}-stimulated ATPase isolated from both 4°C and 25°C-acclimated crayfish muscle had a mean specific activity, at 25°C, of 1.149 uMoles. phosphate liberated mg. protein $^{-1}$. min.$^{-1}$ (average of 31 preparations, Table 7-1). This is amongst the highest activities reported for the microsomal ATPase of a preparation purified simply by differential centrifugation (Table 7-10). Microsomal preparations isolated from mammalian skeletal and cardiac muscle using similar techniques usually have reported activities (at 25°C) of between 0.2-0.6uMoles. phosphate liberated mg. protein^{-1}.

min.$^{-1}$. Extensive purification procedures have been reported to boost this to 31.6uMoles. phosphate liberated mg. protein^{-1}. min.$^{-1}$ in rabbit skeletal muscle (MacLennan 1970) and 3.1uMoles. phosphate liberated. mg. protein^{-1}. min.$^{-1}$ in lobster abdominal muscle extracts (Deamer 1973), both assayed at 37°C. The high specific activity of Ca^{2+}-stimulated ATPase of crayfish muscle preparations is probably because, in common with other Macrurans (Van der Kloot 1969, Baskin 1971), the abdominal muscle contains fewer contaminating membrane systems than the corresponding mammalian tissue. For instance, crayfish abdominal muscle is known to have very few mitochondria (Cossins 1973, Brandt, Reuben, Girardier and Grundfest 1965).

Microsomal preparations isolated from 4°C-acclimated crayfish had a slightly higher mean specific activity than preparations from 25°C-acclimated animals (Table 7-1). Although this difference is not quite statistically significant, it may represent some partial compensation by the crayfish to the effects of a lowered environmental temperature. Hazel and Prosser (1970), in a general review of changes in enzyme activity during thermal adaptations, concluded that those enzymes which show the greatest degree of thermal compensation (Precht types 2 and 3) are those involved in generating the energy currency and reducing power required by the cell at all times. The explanation has usually been given in terms of maintaining the relative constancy of energy availability and

synthetic potential within the cell, in the face of a changing environment. Thus the activities of enzymes associated with the Krebs cycle, glycolysis and the hexose mono-phosphate shunt increase dramatically during cold adaptations.

Of interest in the present study was the fact that 25°C-acclimated crayfish muscle yielded on average more microsomal protein per gram wet weight of muscle than 4°C-acclimated crayfish muscle (see Chapter 6). Since the specific activity of the $Ca^{2+}Mg^{2+}$-ATPase from variously were similar, this indicates that warm-acclimated adapted crayfish/animals possess a greater Ca^{2+}-stimulated ATPase activity per gram wet weight of muscle than cold-acclimated crayfish. This may be related to the high locomotary activity of 25°C-acclimated crayfish at 25°C, in contrast to the more torpid state of 4°C-acclimated animals at 4°C. Other examples of inverse or paradoxical compensations (Precht type 5) or absence of compensations (Precht type 4) have been reported but their interpretation has been uncertain. Hazel and Prosser (1970) have suggested that such acclimations may be expected to occur in enzymes involved in degradative process of the cell, where at higher temperatures there will be a greater need for ridding the cell of the results of degenerative processes and products of metabolic breakdown activity.

B. Temperature Dependence of Enzyme Activity

Since it has been demonstrated previously that Km (ATP) for the Ca^{2+}-stimulated ATPase was essentially temperature independent, it is valid to calculate the Arrhenius activation energy (Ea) of the enzyme reaction at substrate concentrations that are not saturating (Dixon and Webb 1964). Under these conditions the reaction velocity at any substrate concentration varies in direct proportion to its rate constant. The results obtained for Ea at different ATP concentrations (Tables 7-3 and 7-4) for microsomal preparations from both $4^{o}C$ and $25^{o}C$-acclimated crayfish, indicate that the variation associated with these quantities are small. It was concluded that the observed Ea for each preparation was a reproducible quantity which can be measured with a certain degree of accuracy.

The observed variability in Ea between different microsomal preparations of similar adaptational histories (Tables 7-5, 7-6 and 7-7) was therefore taken to be the result of some variability in the preparations and not the analytical technique. Such variability in Ea is a feature that receives scant comment in the literature but was recognised by Inesi and Watanabe (1967) and Suko (1973). It is suggested that comparisons of Arrhenius activation energies between enzyme preparations from different sources and having undergone different treatments, etc., should not be made on the basis of experiments using one preparation,

but should be statistical comparisons of the mean $E_a \pm$S.E.M. of several preparations (cf. Hochachka and Lewis 1971).

The linearity of the Arrhenius plots indicates that there is no thermotropic transition of the membranous lipids, such as has been observed in rabbit white skeletal muscle FSR (Inesi, Millman and Eletr 1973) and Lobster abdominal muscle FSR (Deamer 1973). This is consistent with the observation that purified phospholipids of crayfish muscle are highly unsaturated and undergo a gel to liquid-crystalline phase transition as determined by differential scanning calorimetry at temperatures below 0°C (Cossins, unpublished observations).

C. Effect of Temperature Acclimation upon Activation Energy

A major feature of these results is that there is no difference in temperature characteristics between microsomal preparations from 4°C and 25°C-acclimated crayfish. This is interpreted as being additional evidence that identical enzymes are involved in ATP hydrolysis at both adaptation temperatures. Furthermore there appears to be no modulation of their kinetic behaviour to suit the environmental temperature regime experienced by the animal.

D. Evolutionary Comparisons with other Preparations

It has been proposed by Vroman and Brown (1963) that seasonal and evolutionary adaptation to a cold environment may favour the production of enzymes which can lower the Arrhenius activation energy

(Ea) to minimal levels. Thus in systems with limited thermal energy available there would be a selective advantage in possessing an enzyme which could reduce the energy barriers of the reaction, increase their catalytic efficiency and thereby compensate for the effects of reduced environmental temperatures. Although the interpretation of Arrhenius plots is faught with uncertainties, particularly if they are non-linear (see Hochachka and Somero 1971), such positive relationships between the normal environmental temperature of an organism and the Ea of a particular enzyme reaction, have been reported for pyruvate kinase (Hochachka and Somero 1968), 1969); fructose diphosphatase (Behrisch and Hochachka 1969a, 1969b); muscle glycogen phosphorylase (Assaf and Graves, 1969); succinic dehydrogenase (Vroman and Brown 1963, Somero, Giese and Wohlschlag 1968, Hazel 1972); fructose-diphosphate aldolase (Kwon and Olcott 1965) and for glyceraldehyde-3-phosphate dehydrogenase (Cowey 1967, Greene and Feeney 1970, Feeney, Vanderheede and Osuga 1972). In addition, Smith (1973) has observed conspicuous differences in the activation energy of certain mitochondrial enzyme systems of homoeotherms and poikilotherms, particularly at temperatures between $0^{\circ}C$ and $20^{\circ}C$. These differences in temperature characteristics are thought to be mediated mainly by the nature of the lipid portion of the mitochondrial membrane.

The Ea of 15.57 Kcals.mole^{-1} (65.19 KJ. mole^{-1}.) (average of 14 preparations, measured at 30mM ATP; Table 7-8) for crayfish FSR is very similar to the 16 Kcals.mole^{-1} (66.99 KJ.mole^{-1}) reported for rabbit skeletal muscle FSR by Inesi, Millman and Eletr (1973) and Inesi and Watanabe (1967). However, there is considerable confusion in the literature concerning the temperature characteristics of the Ca^{2+}-stimulated ATPase of rabbit skeletal muscle FSR. Sreter (1969) and Yamamoto and Tonomura (1967) have reported linear Arrhenius plots for this preparation over the temperature range 0-37^{o}C with an activation of 19.0 Kcals.mole^{-1} (79.5 KJ.mole^{-1}.) and 24.0 Kcals.mole^{-1} (100.48 KJ.mole^{-1}) respectively. Inesi and Watanabe (1967) have reported an Ea of between 13.8 and 16.0 Kcal. mole^{-1} (57.57 - 66.99 KJ. mole^{-1}) over the temperature range 5-20^{o}C.

On the other hand Inesi, Millman and Eletr (1973) and Madeira, Antunes-Madeira and Carvalho (1974) have observed a break in the Arrhenius plots of the Ca^{2+}-stimulated ATPase of rabbit muscle FSR occurring at approximately 20^{o}C and 17^{o}C respectively. These workers disagree over the value of Ea both above and below the temperature of transition. Madeira, Antunes-Madeira and Carvalho (1974) have suggested that such discrepancies may have methodological explanations.

The Ca^{2+}-stimulated ATPase of lobster FSR is reported to undergo a transition at either 17^{o}C (Deamer 1973) or 11.5^{o}C (Madeira, Antunes-Madeira and Carvalho 1974). Ea at temperatures above the transition were estimated at 6 or 8.7 Kcal.

mole^{-1}. (25.12 or 36.42 KJ. mole^{-1}), whilst below the transition, the activation energy becomes approximately 20 Kcal. mole^{-1}. This is rather surprising since the lobster would have solid, gel membrane at its normal environmental temperature, in contrast to current views that the liquid-crystalline state of the membrane is essential to its proper functioning (McElhaney 1974). By contrast there is no evidence for a similar transition occurring in the Ca^{2+}-stimulated ATPase activity of the FSR isolated from cold- or warm-acclimated crayfish. It is not possible to make firm conclusions from such confused data.

However, this relationship between Ea and environmental temperature does not hold for all enzymes studied, particularly in temperate animals. (Molluscan ribonucleases, Read 1964; lactate dehydrogenase, Hochachka and Somero 1968; acetyl cholinesterase, Baldwin and Hochachka 1970) and in some cold-adapted species (see Feeney, Vandenheede and Osuga 1972). But as discussed by Somero (1969) this does not necessarily invalidate the general hypothesis, since there are good reasons for expecting the selection pressure for lowered Ea during cold adaptation to vary amongst different enzymes. Firstly, those enzymes which are rate-limiting and irreversible, such as pyruvate kinase, would be subject to intense selective pressure, relative to an enzyme which is not rate-limiting. Indeed, Johnson (1971) has demonstrated a correlation between the equilibrium (Keq) constant

of an enzyme and an index of the extent of polymorphism in that enzyme. This suggests that rate limiting enzymes, which are essentially irreversible (i.e., high Keq.), are responsive to evolutionary selection pressure. Secondly, reductions in Ea may cause simultaneous alterations in the kinetic and/or regulatory properties of the enzymes which may be deleterious. The various kinetic properties of an enzyme, therefore, may be a compromise between the several possible results of selection pressure. Thirdly, it may prove impossible to reduce Ea for some enzymic reactions below certain threshold levels.

The data quoted by Hochachka and Somero (1973) in support of this hypothesis shows that the free energy of activation (ΔG^*; the asterisk indicates a parameter of the activation stage) for any particular enzymic reaction is very similar for homologous enzymes from both poikilotherms and homoeotherms. However, there is a major reduction in the change in activation enthalpy (ΔH^*) and activation entropy (ΔS^*) during activation in poikilothermic lactate dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase and phosphorylase-b (Hochachka and Somero 1973). The reduction in ΔH^* probably represents the reduced heat content of the localised environment, and it would appear that a major strategy during evolutionary cold adaptation is the production of enzymes with a reduced activation entropy change (ΔS^*) which compensates for reduced ΔH^*.

Indeed LDH and glyceraldehyde-3-phosphate dehydrogenase actually decrease their entropy during activation (Hochachka and Somero 1973). It is evident from the fundamental thermodynamic equation -

$$\Delta G^* = \Delta H^* - T\Delta S^*$$

where T is the absolute temperature, that a simultaneous reduction in ΔH^* and ΔS^* would result in the constancy of ΔG^* (see Inesi, Millman and Eletr 1973).

As indicated earlier, modulation of ΔS^* for an enzyme reaction is a property which might also affect other kinetic or regulatory parameters. A reduction in ΔS^* may be attained only at the expense of reduced efficiency at other stages of the enzyme-mediated reaction, such as conformational flexibility and interaction of the enzyme-substrate or enzyme-modulator complex. Evolutionary selection may be expected therefore, to balance changes in ΔS^* with other important kinetic or regulatory parameters to attain the most successful compromise.

Table 7-1: Comparison of mean Ca^{2+}-stimulated ATPase and mean Mg^{2+}-dependent ATPase activities of muscle microsomal preparations isolated from 4°C and 25°C acclimated crayfish

Extraction Procedure

See 'Materials and Methods' and Chapter 5.

Assay Conditions

Mg^{2+}-dependent ATPase activity was assayed in duplicate at 25 ± 0.1°C in the presence of 0.5mM EGTA, 3mM $MgCl_2$, 75mM sucrose, 100mM Kcl, 25mM Imidazole-HCl pH 7.1 and 3mM Tris-ATP (all final concentrations) in a final volume of 2ml.

Total Ca^{2+}-Mg^{2+}-ATPase was assayed in duplicate at 25°C in the same medium but in the presence of 0.5mM $CaCl_2$.

Ca^{2+}-stimulated ATPase activity is defined as the difference between the ATP hydrolysis in the presence of both calcium and magnesium, and ATP hydrolysis in the presence of magnesium only.

Phosphate and Protein Assay

See Chapter 5.

Legend

n - number of preparations assayed

P - probability for (n-2) degrees of freedom

N.S. - not significant

Table 7-1

Activity	Ca^{2+}-stimulated-ATPase		Mg^{2+}-dependent ATPase	
Adaptation Temperature (^{o}C)	4	25	4	25
$\overline{X}$ (uM Pi.mg.protein^{-1}.min^{-1})	1.311	0.988	1.108	0.061
S.E.M.	0.073	0.160	0.021	0.024
n	17	14	4	3
t	1.838		1.479	
P	0.10-0.05		0.2-0.1	
Significance	N.S.		N.S.	

Table 7-2: Comparison of the Arrhenius activation energy calculated from Vmax at high and low ranges of ATP concentration of preparations isolated from $4^{o}C$ and $25^{o}C$ acclimated crayfish

Extraction Procedure

See 'Materials and Methods' and Chapter 5.

Assay Conditions

Original data is presented in Tables 6-6 to 6-9. Ca^{2+}-stimulated activity was assayed at temperatures between $0^{o}C$ and $40^{o}C$ as detailed in Chapter 5. See also Table 7-1.

The activation energy Ea, was determined by regression analysis of the data (Snedecor and Cochran, 1967).

Phosphate and Protein Assay

See Chapter 5.

Legend

r - correlation coefficient

n - number of analyses

P - probability for (n-2) degrees of freedom

H.S. - highly significant

Table 7-2:

Adaptation Temperature ^{o}C	4		25	
Range of ATP concentrations used for activity determination	0.1-0.5	0.5-3.0	0.1-0.5	0.5-3.0
Slope b	-3.63	-3.47	-3.44	-3.10
Ea (Kcal.mole^{-1})	16.63	15.90	15.74	14.19
Ea (KJoule mole $^{-1}$)	69.6	66.6	63.1	59.4
r	-0.9957	-0.9964	-0.9905	-0.9932
n	8	8	8	8
P	$<$0.001	$<$0.001	$<$0.001	$<$0.001
Significance of Correlation	H.S.	H.S.	H.S.	H.S.

Table 7-3: Regression data for Arrhenius plots of Ca^{2+}-stimulated ATPase activity for a muscle microsomal preparation isolated from $4^{o}C$ acclimated crayfish at different ATP concentrations

Extraction Procedure

See Chapter 5

Assay Conditions

The original data is presented in Tables 6-4 and 6-5.

The activation energy Ea, at each concentration of ATP was determined by regression analysis of the data (Snedecor and Cochran, 1967).

Phosphate and Protein Analysis

See Chapter 5.

Legend

r - correlation coefficient

n - number of observations

P - probability for (n-2) degrees of freedom

H.S. - highly significant

Table 7-3

ATP concentration (mM)	Regression Data				n	P	Significance or correlation
	Slope b	Ea ($Kcal.mole^{-1}$)	Ea ($KJoule\ mole^{-1}$)	r			
3.00	-3.48	15.91	66.61	-0.9969	8	<0.001	H.S.
1.50	-3.50	16.03	67.11	-0.9979	8	<0.001	H.S.
1.00	-3.54	16.20	67.82	-0.9972	8	<0.001	H.S.
0.50	-3.53	16.17	67.70	-0.9973	16	<0.001	H.S.
0.30	-3.57	16.36	68.49	-0.9967	8	<0.001	H.S.
0.20	-3.52	16.12	67.49	-0.9983	16	$\ll 0.001$	H.S.
0.16	-3.57	16.34	68.41	-0.9967	8	<0.001	H.S.
0.13	-3.46	15.85	66.36	-0.9982	8	<0.001	H.S.
0.10	-3.46	15.83	66.28	-0.9955	16	$\ll 0.001$	H.S.

Table 7-4: Regression data for Arrhenius plots of Ca^{2+}-stimulated ATPase activity for a muscle microsomal preparation isolated from 25°C adapted crayfish at different ATP concentrations

Extraction Procedure

See Chapter 5

Assay Conditions

The original data is presented in Tables 6-4 and 6-5.

The activation energy Ea, at each concentration of ATP was determined by regression alaysis of the data (Snedecor and Cochran, 1967).

Phosphate and Protein Analysis

See Chapter 5

Legend

r - correlation coefficient

n - number of observations

P - probability for (n-2) degrees of freedom

H.S. - highly significant

Table 7-4

ATP concentration (uM)	Regression Data				n	P	Significance of correlation
	Slope b	Ea (Kcal.mole^{-1})	Ea (KJoule mole^{-1})	r			
3.00	-3.23	14.80	61.96	-0.9968	8	<0.001	H.S.
1.50	-3.30	15.11	63.29	-0.9949	8	<0.001	H.S.
1.00	-3.32	15.18	63.55	-0.9934	8	<0.001	H.S.
0.50	-3.14	14.36	60.12	-0.9989	7	<0.001	H.S.
0.30	-3.40	15.55	65.10	-0.9982	8	<0.001	H.S.
0.20	-3.15	14.44	60.46	-0.9930	16	$\ll 0.001$	H.S.
0.16	-3.23	14.76	61.80	-0.9967	8	<0.001	H.S.
0.13	-3.27	14.95	62.59	-0.9972	9	<0.001	H.S.
0.10	-3.24	14.82	62.05	-0.9936	15	$\ll 0.001$	H.S.

Table 7-5: Regression Data for Arrhenius plot of Ca^{2+}-stimulated ATPase activity of several microsomal preparations isolated from 4°C and 25°C adapted crayfish muscle at 0.5mM ATP

Extraction Procedure

See Chapter 5

Assay Conditions

The Ca^{2+}-stimulated ATPase activity was assayed at temperatures between 0-40°C (see 'Materials and Methods') using an aluminium Forbes bar.

The activation energy Ea, for each preparation was determined by regression analysis of the data (Snedecor and Cochran, 1967).

Phosphate and Protein Analysis

See Chapter 5

Legend

r - correlation coefficient

n - number of observations

P - probability for (n-2) degrees of freedom

H.S. - highly significant

Table 7-5

Adaptation Temperature °C	Preparation Number	Regression Data				n	P	Significance of correlation
		Slope b	Ea (Kcal.mole^{-1})	Ea (KJoule mole^{-1})	r			
4	1	-3.598	16.46	68.91	-0.9960	8	<0.001	H.S.
4	2	-3.514	16.08	67.32	-0.9921	6	<0.001	H.S.
4	3	-2.874	13.15	55.06	-0.9780	16	$\ll 0.001$	H.S.
4	4	-3.280	15.01	62.84	-0.9940	8	<0.001	H.S.
4	5	-3.480	15.92	66.65	-0.9920	7	<0.001	H.S.
4	6	-3.630	16.61	69.54	-0.9970	7	<0.001	H.S.
25	7	-3.390	15.51	64.90	-0.9970	8	<0.001	H.S.
25	8	-3.371	16.98	71.09	-0.9890	8	<0.001	H.S.

Table 7-6: Regression data for Arrhenius plots of Ca^{2+}-stimulated ATPase activity of several microsomal preparations isolated from 4°C acclimated crayfish muscle at 3.0mM ATP

Extraction Procedure

See Chapter 5

Assay Conditions

The Ca^{2+}-stimulated ATPase activity was assayed at temperatures between 0-40°C (see 'Materials and Methods') using an aluminium Forbes bar.

The activation energy Ea, for each preparation was determined by regression analysis of the data (Snedecor and Cochran, 1967).

Phosphate and Protein Analysis

See Chapter5

Legend

r - correlation coefficient

n - number of observations

P - probability for (n-2) degrees of freedom

H.S. - highly significant

Table 7-6

Adaptation Temperature °C	Preparation Number	Regression Data				n	P	Significance of Correlation
		Slope b	Ea (Kcal.mole^{-1})	Ea (KJoule mole^{-1})	r			
4	1	-3.478	17.33	72.56	-0.9930	8	$<$0.001	H.S.
4	2	-3.062	14.01	58.66	-0.9920	8	$<$0.001	H.S.
4	3	-3.617	16.55	69.29	-1.000	3	$<$0.001	H.S.
4	4	-3.200	14.64	61.29	-0.9983	8	$<$0.001	H.S.
4	5	-3.420	15.65	65.48	-0.9984	3	$<$0.02	S
4	6	-3.480	15.92	66.65	-0.9840	7	$<$0.001	H.S.
4	7	-3.140	14.37	60.16	-0.9960	8	$<$0.001	H.S.
4	8	-3.514	16.24	67.99	-0.9836	6	$<$0.001	H.S.
4	9	-3.452	15.79	66.11	-0.9895	13	$<$0.001	H.S.

Table 7-7: Regression data for Arrhenius plots of Ca^{2+}-stimulated ATPase activity of several microsomal preparations isolated from 25°C acclimated crayfish muscle at 3.0mM ATP

Extraction Procedure

See Chapter 5

Assay Conditions

The Ca^{2+}-stimulated ATPase activity was assayed at temperatures between 0-40°C (see 'Materials and Methods') using an aluminium Forbes bar.

The activation energy Ea for each preparation was determined by regression analysis of the data (Snedecor and Cochran, 1967).

Phosphate and Protein Analysis

See Chapter 5

Legend

r - correlation coefficient

n - number of observations

P - probability for (n-2) degrees of freedom

H.S. - highly significant

Table 7-7

Adaptation Temperature ^{o}C	Preparation Number	Regression Data				n	P	Significance of correlation
		Slope b	Ea (Kcal.mole^{-1})	Ea (KJoule mole^{-1})	r			
25	1	-3.56	16.29	68.20	-0.9765	18	$<$0.001	H.S.
25	2	-3.41	15.60	65.31	-0.9920	8	$<$0.001	H.S.
25	3	-3.176	14.53	60.83	-0.9900	8	$<$0.001	H.S.
25	4	-3.690	16.88	70.67	-0.9935	9	$<$0.001	H.S.
25	5	-3.083	14.11	59.07	-0.9824	10	$<$0.001	H.S.

Table 7-8: Comparison of the mean Arrhenius activation energy of the microsomal Ca^{2+}-stimulated ATPase preparations isolated from 4°C and 25°C acclimated crayfish, measured at 0.5mM and 3.0mM ATP

Extraction Procedure

See Chapter 5

Assay Conditions

The Ca^{2+}-stimulated ATPase activity was assayed at temperatures between 0-40°C (see 'Materials and Methods') using an aluminium Forbes bar.

The activation energy Ea, for each preparation was determined by regression analysis of the data (Snedecor and Cochran, 1967: see Tables 7-5, 7-6 and 7-7 for details).

Phosphate and Protein Analysis

See Chapter 5

Legend

Ea - mean activation energy

n - number of preparations assayed

P - probability for (n-2) degrees of freedom

N.S. - not significant

Table 7-8

ATP concentration (mM)	3.0		0.5	
Adaptation Temperature ^{o}C	4	25	4	25
Ea (Kcal. $mole^{-1}$) (Range)	15.61 (14.01-17.33)	15.48 (14.11-16.88)	15.53 (13.15-16.61)	16.25 (15.51-16.98)
S.E.M.	0.346	0.520	0.453	2.12
n	9	5	7	2
t	0.208		0.282	
P	0.8-0.9		0.7-0.8	
Significance	N.S.		N.S.	

Table 7-9: Comparison of the mean activation energy of Ca^{2+}-stimulated activity of muscle microsomal preparations from 4°C acclimated crayfish at .5mM and 3.0mM ATP

Extraction Procedure

See Chapter 5

Assay Conditions

The Ca^{2+}-stimulated ATPase activity was assayed at temperatures between 0-40°C (see 'Materials and Methods') using an aluminium Forbes bar.

The activation energy Ea for each preparation was determined by regression analysis of the data (Snedecor and Cochran, 1967; see Tables 7-5 and 7-6).

Phosphate and Protein Analysis

See Chapter 5.

Legend

n - number of preparations assayed

P - probability for (n-2) degrees of freedom

N.S. - not significant

Table 7-9

ATP concentration (mM)	3.0	0.5
Ea ($Kcal.mole^{-1}$) (Range)	15.61 (14.01-17.33)	15.53 (13.15-16.61)
S.M.E.	0.346	0.453
n	9	7
d.f = (n-2)	14	
t	0.0442	
P	>0.9	
Significance	N.S.	

Table 7 - 10. Summary of data from the literature concerning the the reaction constants for the calcium uptake system from various tissues.

Source of Microsomal Preparation	Activity Monitored	Km (uM)	Vmax (uM Pi . mg protein^{-1}. mm^{-1}.)	Reference
Rabbit White Skeletal Muscle	Ca^{2+} stimulated ATPase ATP	0.7 30	1.14* 3.0	Yamamoto and Tonomura (1967)
Rabbit White Skeletal Muscle	Phosphorylation of sarcoplasmic reticulum membrane P_{32}	1.6 ?	? ?	Inesi, Maring Murphy and McFarland (1970)
Rabbit White Skeletal Muscle	Ca^{2+} - stimulated ATPase ATP	10 > 1000	1.7 0.6	Inesi, Goodman and Watanabe (1967)
Human Erythrocyte Membrane	Ca^{2+} ATPase Ca^{2+}	4 100	- 66% - 33%	Schatzmann and Rossi (1971)
Crayfish Abdominal Muscle	Ca^{2+} - stimulated ATPase ATP	70 - 80 315 - 347	- 66% - 33%	Present work
Rabbit White Skeletal Muscle	Ca^{2+} stimulated ATPase ATP	1.0 ?	? ?	Horgan (1974)

Table 7-11: Summary of the literature concerning the EA's of Ca^{2+}-stimulated ATPase and Ca^{2+} uptake of sarcoplasmic reticulum from various sources

Preparation	Function Measured	Temperature Range (°C)	Ea (Kcal. $mole^{-1}$)	Ea (KJoule $mole^{-1}$)	Reference
Rabbit White Skeletal Muscle	Ca^{2+}-stim. ATPase	20 20	16 29	67 121	Inesi, Millman and Eletr (1973)
"	Ca^{2+} uptake	20 20	17 28	71 117	
Rabbit White Skeletal Muscle	Ca^{2+}-stim. ATPase	0-37	24.0 26.2	100 109.7	Yamamoto and Tonomura (1967)
Rabbit White Skeletal Muscle	Ca^{2+}-stim. ATPase	6-20	13.8-16.0	57.8-66.9	Inesi and Watanabe (1967)
Rabbit White Skeletal Muscle	Ca^{2+}-stim. ATPase	0-35	19.0	79.5	Sreter (1969)
Rabbit Red Skeletal Muscle	Ca^{2+}-stim. ATPase	0-35	11.2	46.9	
Rabbit Skeletal Muscle	Ca^{2+}-stim. ATPase	2-16.7 16.7-37	19.4 10.1	81.2 42.3	Maderia, Antunes-Madeira, Carvalho (1974)
Lobster Muscles	Ca^{2+}-stim. ATPase	2-11.5 11.5-37	19.7 8.7	82.5 36.4	
Lobster Muscles	Ca^{2+}-stim. ATPase	0-17 17-25	20 6	83.7 25.1	Deamer (1973)
Crayfish abdominal Muscle	Ca^{2+}-stim. ATPase	0-40	15.6*	65.3*	Present Study

* See Table 7-8

Figure 7-1: Temperature dependence of Vmax for the microsomal Ca^{2+}-stimulated ATPase from the muscle of 4°C acclimated crayfish

Method: Vmax at each temperature was calculated from Lineweaver-Burk plots for activity at high (Table 6-6) and low ranges (Table 6-7) of ATP concentration.

Legend: Ordinate: Activity (μM Pi. mg protein^{-1})

Abscissa: $1/T^{o}K \times 10^{3}$

O Vmax at high (ATP)

O Vmax at low (ATP)

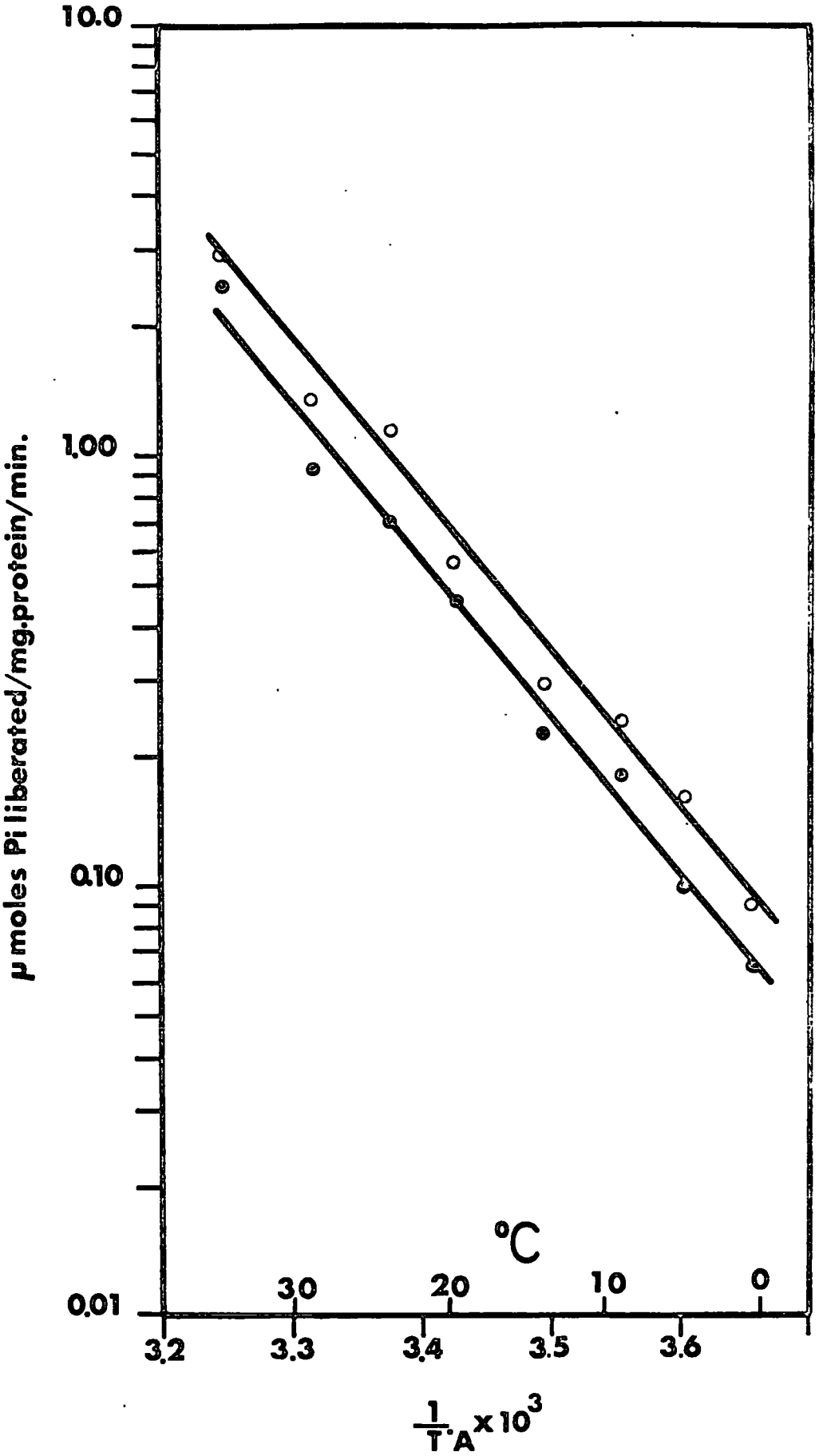

10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min.
°C
30
20
10
0
3.2
3.3
3.4
3.5
3.6
1/T·A x 10³

Figure 7-2: Temperature dependence of Vmax for the microsomal Ca^{2+}-stimulated ATPase from the muscle of 25°C acclimated crayfish

Methods: Vmax at each temperature was calculated from Lineweaver-Burk plots for activity at high (Table 6-8) and low ranges (Table 6-9) of ATP concentration.

Legend: Ordinate: Activity (μM Pi. mg protein^{-1}. minute^{-1})

Abscissa: $1/T^{o}K \times 10^{3}$

o Vmax at high (ATP)

o Vmax at low (ATP)

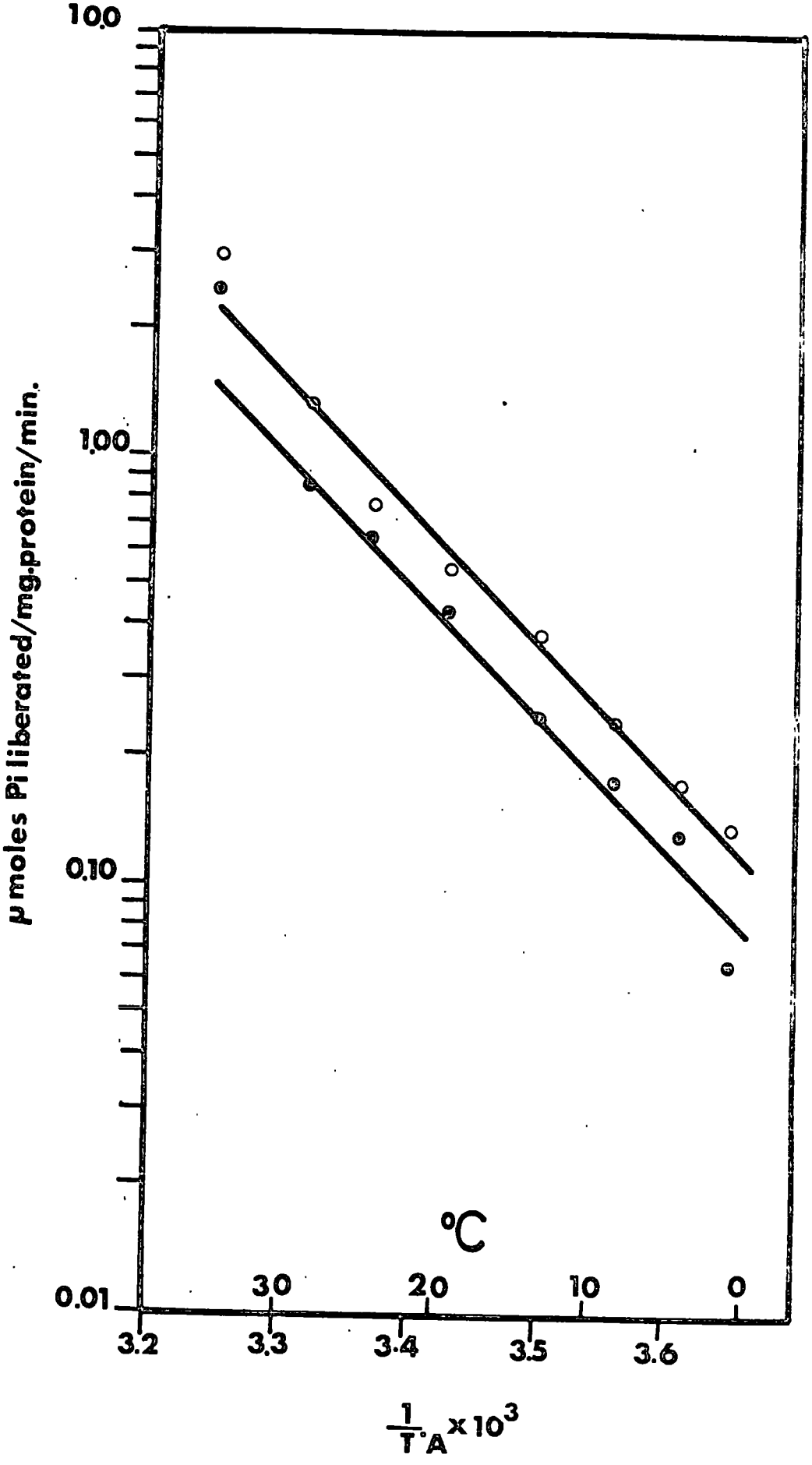

10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min.
°C
30
20
10
0
3.2
3.3
3.4
3.5
3.6
1/T°A x 10³

Figure 7-3: The effect of temperature upon the activity of the Ca^{2+}-stimulated ATPase at different ATP concentrations, for a microsomal preparation isolated from 4°C acclimated crayfish

Methods: Basic data is presented in Table 6-4

Legend: Ordinate: Activity
(μM Pi. mg protein^{-1}. minute^{-1})

Abscissa: Temperature °C
The ATP concentrations are indicated on the Figure

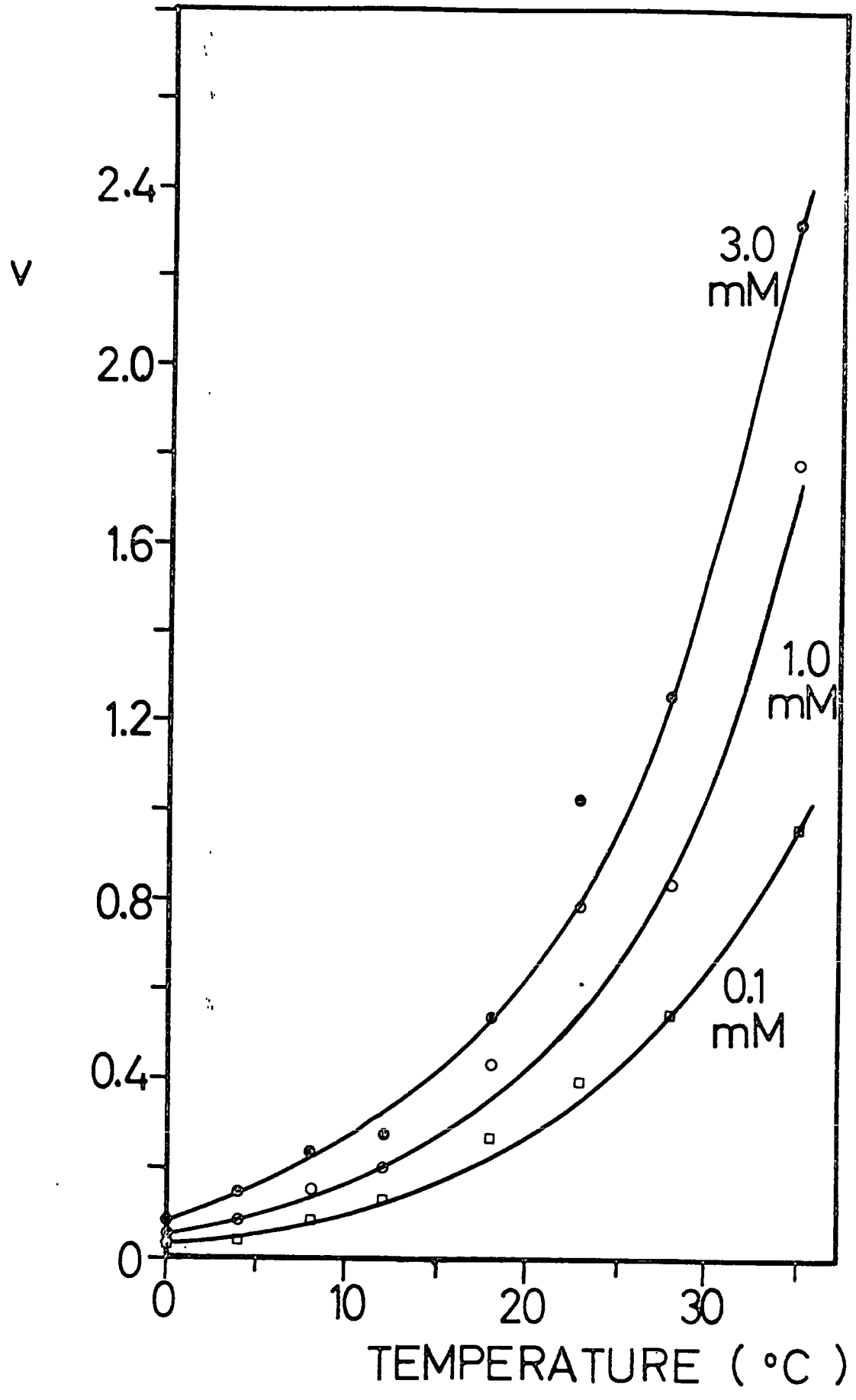

V
2.4
2.0
1.6
1.2
0.8
0.4
0
0
10
20
30
TEMPERATURE (°C)
3.0 mM
1.0 mM
0.1 mM

Figure 7-4: The effect of temperature upon the activity of the Ca^{2+}-stimulated ATPase at different ATP concentrations, for a microsomal preparation isolated from 25°C acclimated crayfish

Methods: Basic data is presented in Table 6-4

Legend: Ordinate: Activity
(μM Pi mg protein^{-1}. minute^{-1}.)

Abscissa: Temperature °C
The ATP concentrations are indicated on the Figure

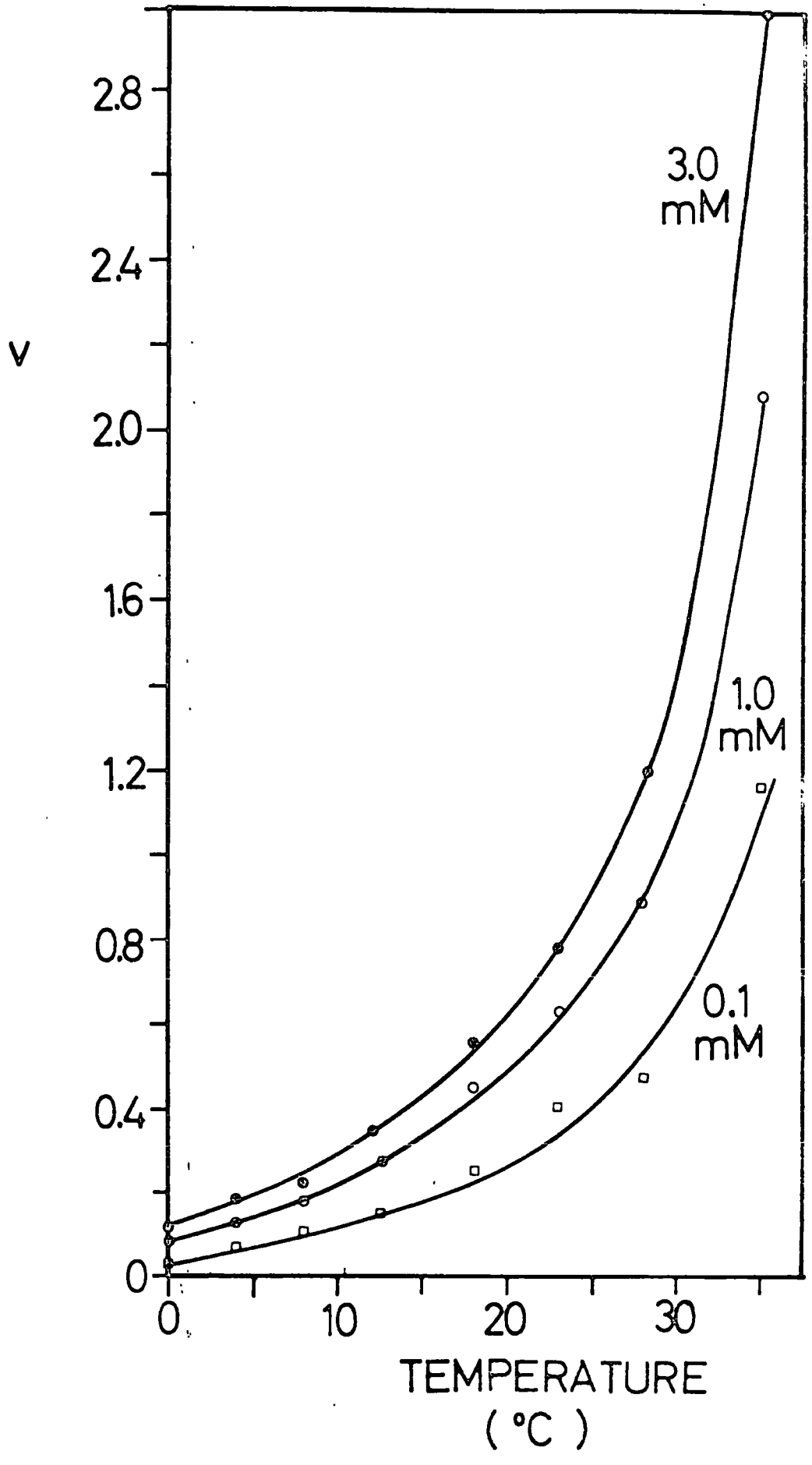

V
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
0
10
20
30
TEMPERATURE
(°C)
3.0
mM
1.0
mM
0.1
mM

Figure 7-5: Arrhenius plots of the activity of the Ca^{2+}-stimulated ATPase activity at different ATP concentrations for a microsomal preparation isolated from $4^{o}C$ acclimated crayfish

Methods: See Figure 7-3

Legend: Ordinate: Activity

(μM Pi. mg protein^{-1}. minute^{-1}.)

Abscissa: $1/T^{o}K \times 10^{3}$

△ 3.00 mM ATP

□ 1.00 mM ATP

○ 0.30 mM ATP

⊙ 0.10 mM ATP

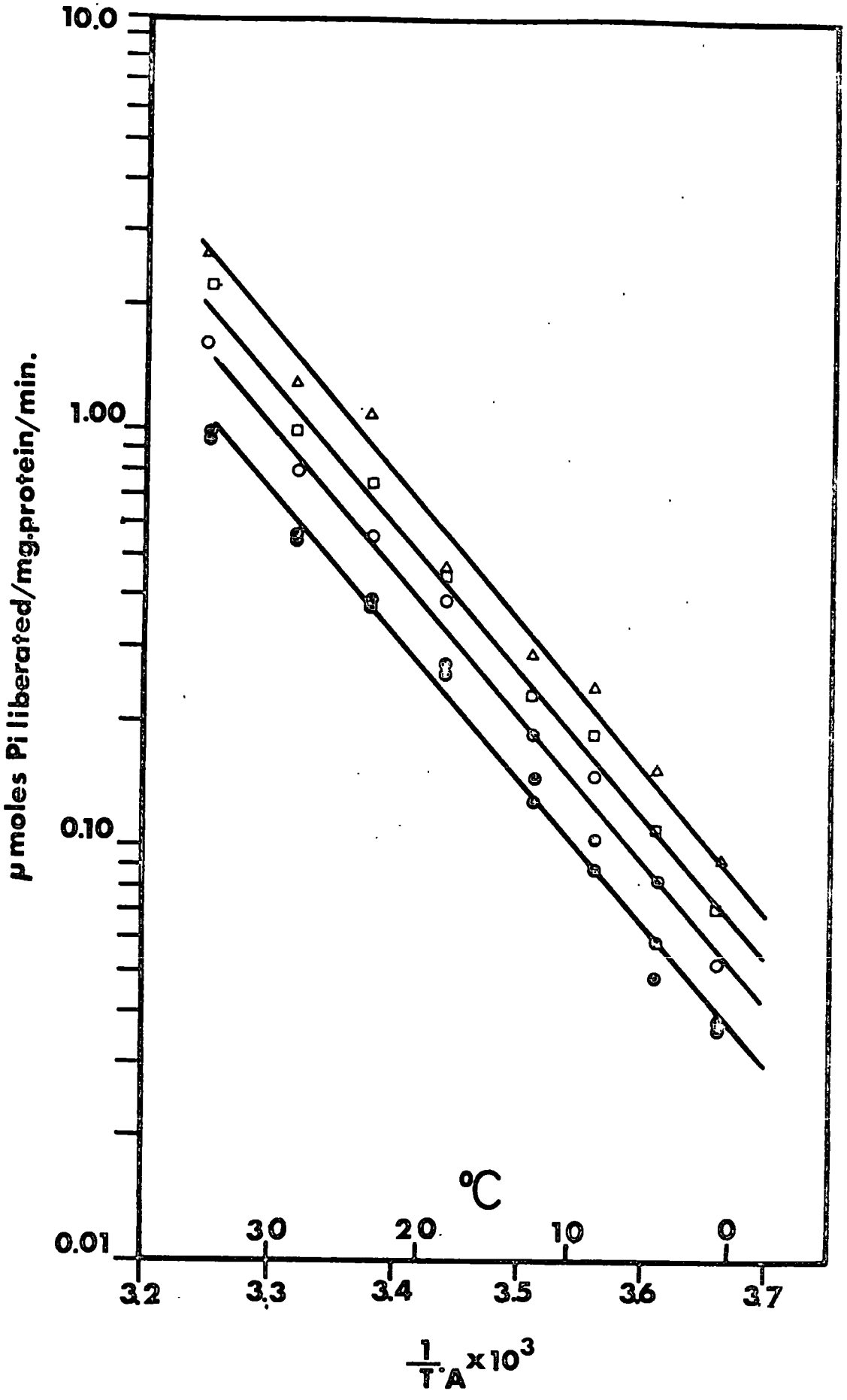

10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min.
°C
30
20
10
0
3.2
3.3
3.4
3.5
3.6
3.7
$\frac{1}{T}$ A x 10³

Figure 7-6: The Arrhenius plots of the activity of the Ca^{2+}-stimulated ATPase activity at different ATP concentrations for a microsomal preparation isolated from 25°C acclimated crayfish

Methods: See Figure 7-4

Legend: Ordinate: Activity

(μM Pi. mg protein^{-1}. minute^{-1}.)

Abscissa: 1/T°K x 10^3

△ 3.00 mM ATP

□ 1.00 mM ATP

○ 0.30 mM ATP

◘ 0.10 mM ATP

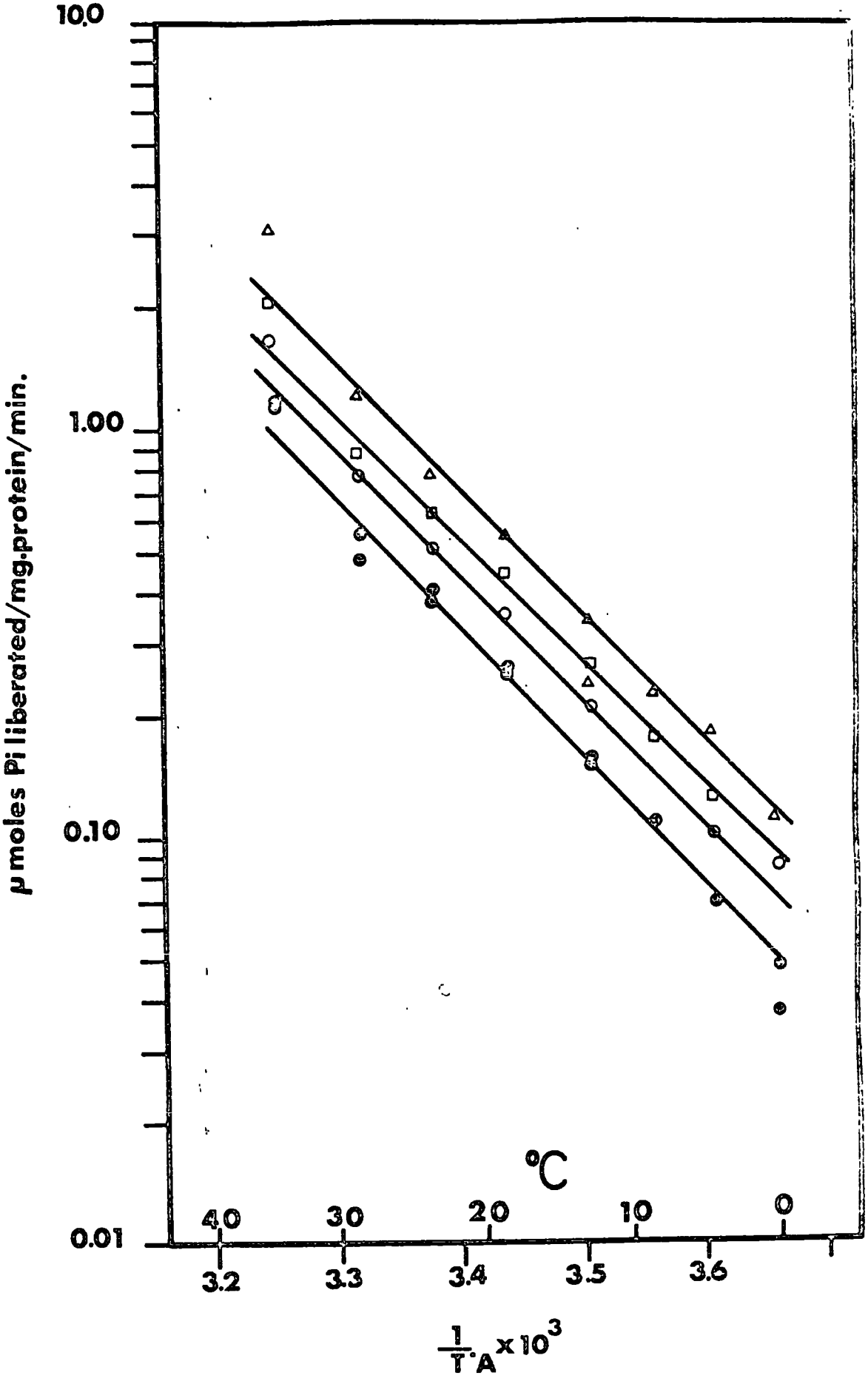

10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min.
°C
40
30
20
10
0
3.2
3.3
3.4
3.5
3.6
1/T·A x 10³

Figure 7-7: Temperature dependence of the Ca^{2+}-stimulated ATPase activity at 3.0 mM ATP of several microsomal preparations isolated from 4°C acclimated crayfish

Methods: Ca^{2+}-stimulated ATPase activity was assayed in the presence of 3.0 mM ATP at different temperatures using the methods described earlier

Legend: Ordinate: Activity
(μM Pi. mg protein^{-1}. minute^{-1}.)
Abscissa: $1/T^{o}K \times 10^{3}$
Each symbol represents a different preparation

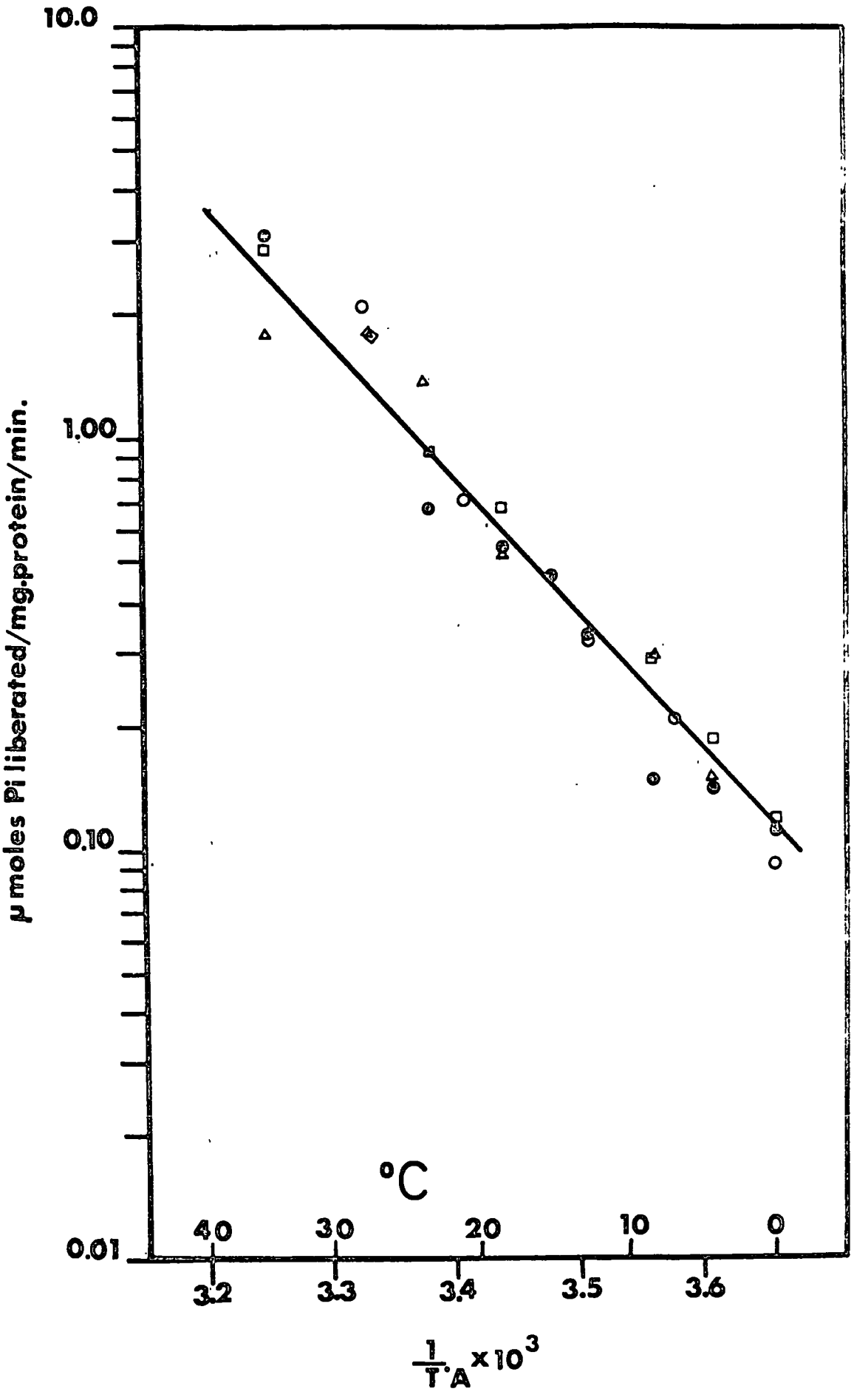

10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min.
°C
40
30
20
10
0
3.2
3.3
3.4
3.5
3.6
1/T°A x 10³

Figure 7-8: Temperature dependence of the Ca^{2+}-stimulated ATPase activity at 0.5 mM ATP of several microsomal preparations isolated from 4°C acclimated crayfish

Methods: Ca^{2+}-stimulated activity was assayed in the presence of 0.5 mM ATP at different temperatures using the methods outlined earlier

Legend: Ordinate: Activity
(μM Pi. mg protein^{-1}. minute^{-1}.)
Abscissa: $1/T^{\circ}K \times 10^{3}$
Each symbol represents a different preparation

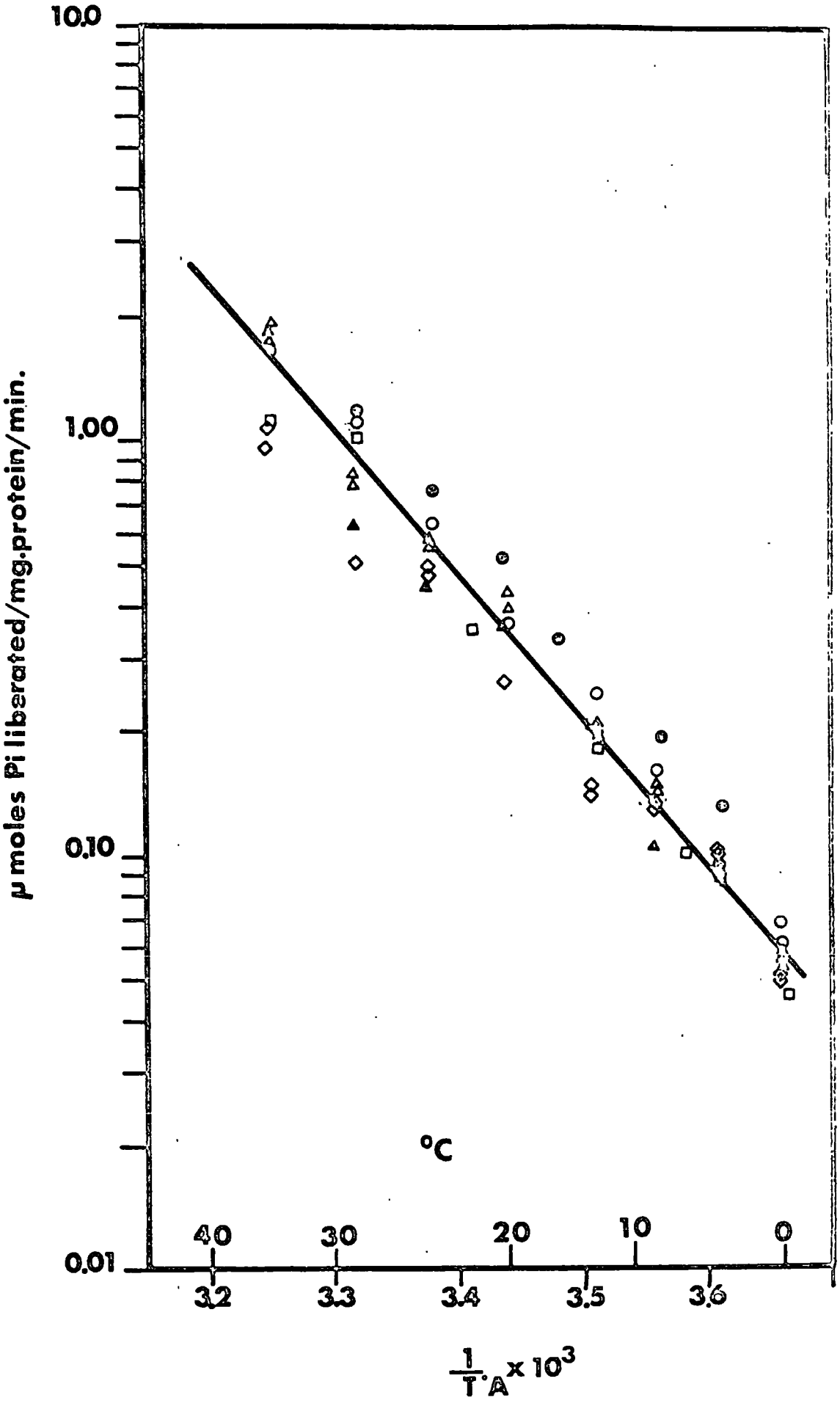

10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min.
°C
40
30
20
10
0
3.2
3.3
3.4
3.5
3.6
1/T·A x 10³

Figure 7-9: Temperature dependence of the Ca^{2+}-stimulated ATPase activity at 3.0 mM ATP of several microsomal preparations isolated from 25°C acclimated crayfish

Methods: Ca^{2+}-stimulated ATPase activity was assayed in the presence of 3.0 mM ATP, at different temperatures using the methods described earlier

Legend: Ordinate: Activity
(μM Pi. mg protein^{-1}. minute^{-1}.)
Abscissa: $1/T^{o}K \times 10^{3}$
Each symbol represents a different preparation

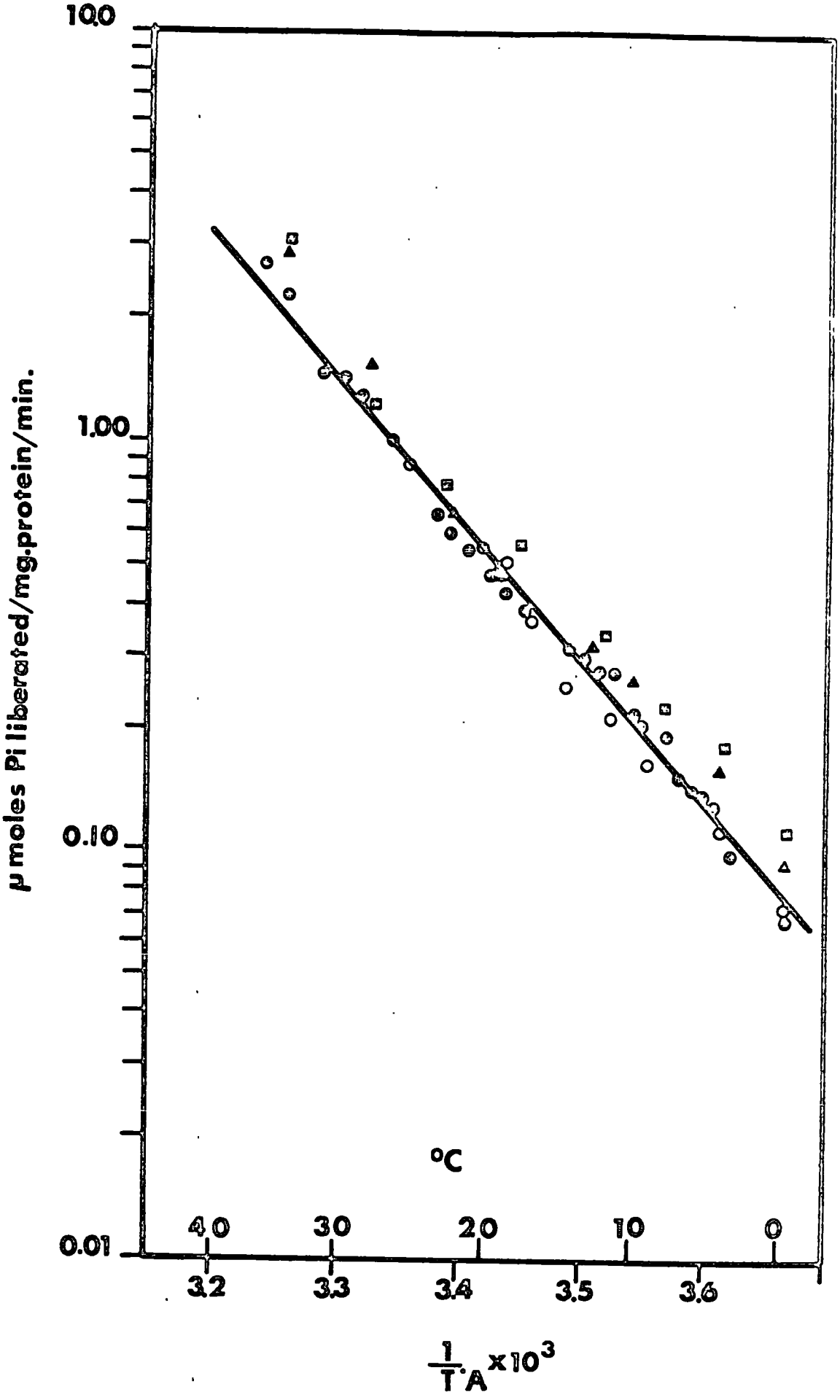

10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min.
°C
40
30
20
10
0
3.2
3.3
3.4
3.5
3.6
1/T·A x10³

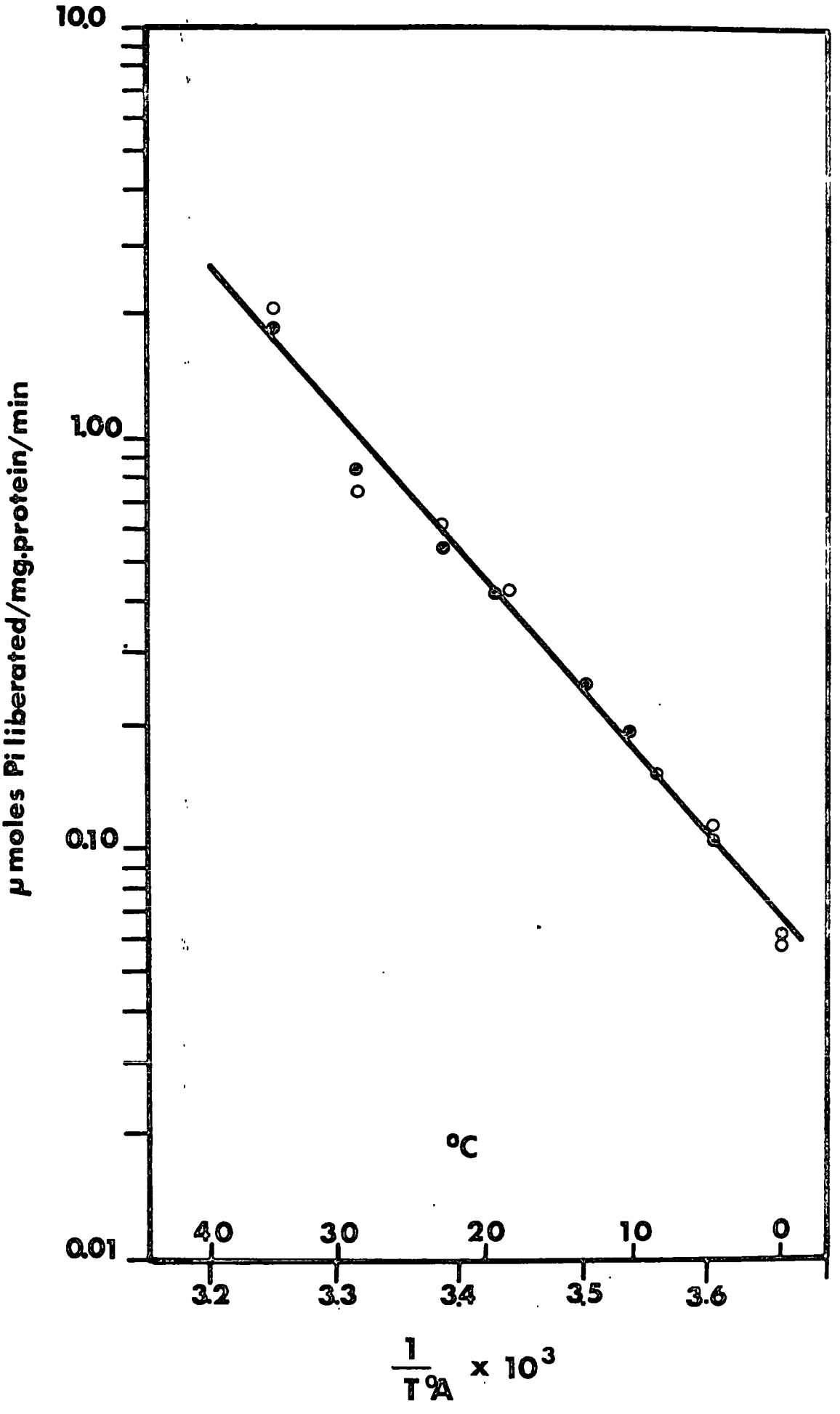
Figure 7-10: Temperature dependence of the Ca^{2+}-stimulated ATPase activity at 0.5 mM ATP of several microsomal preparations isolated from 25°C acclimated crayfish

Methods: Ca^{2+}-stimulated ATPase activity was assayed in the presence of 0.5 mM ATP, at different temperatures using the methods described earlier.

Legend: Ordinate: Activity
(μM Pi. mg protein^{-1}. minute.)

Abscissa: $1/T^{o}K \times 10^{3}$

Each symbol represents a different preparation


10.0
1.00
0.10
0.01
μ moles Pi liberated/mg.protein/min
°C
40
30
20
10
0
3.2
3.3
3.4
3.5
3.6
1/T°A x 10³

Section III

STUDIES ON THE THERMAL INACTIVATION OF MEMBRANE-BOUND ENZYMES

"It may be said at the outset that virtually nothing is known with certainty about the structure of lipoproteins."

Lovern (1954)

Chapter 8

THERMAL INACTIVATION OF THE Ca^{2+}-STIMULATED ATPase FROM CRAYFISH MUSCLE

INTRODUCTION

Ushakov (1964) has propounded the 'protein coagulation' hypothesis of cellular heat injury, although protein denaturation of those "soluble" enzymes studied by this worker and his colleagues occurred only at temperatures well above those responsible for heat death of the whole animal.

However, Bowler and Duncan (1967) have shown that the microsomal Mg^{2+}-dependent ATPase of the freshwater crayfish was markedly inactivated by preincubation at 30-35°C, temperatures that correspond closely to those that cause heat death of the whole animal. Gladwell (1973; see also Bowler, Duncan, Gladwell and Davison, 1973) later confirmed this observation, and further demonstrated that the kinetics of this process were influenced by the temperature of acclimation of the whole animal in a compensatory manner. Thus the enzyme isolated from 10°C acclimated crayfish was 85% inactivated by preincubation at 34°C for 25 minutes, whereas the preparation isolated from 25°C acclimated crayfish was only inactivated by 67%. In addition, Gladwell (1973) has shown that preincubation of whole muscle blocks from 10°C acclimated crayfish at lethal temperatures causes a similar inactivation of the Mg^{2+}-ATPase.

The close correlation noted by Bowler and Duncan (1967), Gladwell (1973) and Bowler, Duncan, Gladwell and Davison (1973) between the temperatures that caused large-scale ionic movements between muscle and the haemolymph, those that caused heat death, and those that caused inactivation *in vitro* of the Mg^{2+} ATPase of a microsomal preparation, led to the suggestion that the membrane-

bound Mg^{2+}-dependent ATPase was involved in the control of passive membrane permeability. It was envisaged by Bowler and Duncan (1967) and by Gladwell (1973) that lethal temperatures caused inactivation of this enzyme, resulting in the loss of passive permeability control and a decline in the trans-membrane ionic gradients. Heat death itself was thought to be caused by the disruptive effects of a high haemolymph K^+ concentration upon the nervous system (Gladwell, 1973).

A major problem in this interpretation is the identity of the Mg^{2+}-ATPase studied by these workers. It is evident from their preparative technique that the 13,000-107,000g microsomal fraction used, will include a variety of enzymes, including the Ca^{2+}-activated ATPase, and Mg^{2+}-dependent ATPase of the sarcoplasmic reticulum, the myofibrillar ATPases and other ATPases associated with sarcotubular elements, as well as the (Na^+, K^+) ATPase and Mg^{2+}-ATPase of the sarcolemma. The absence of EGTA in the isolation or reaction media suggests that Ca^{2+}-activated systems would be activated in the presence of Mg^{2+} alone, by endogenous calcium. The omission of 'salt' extraction (Martonosi and Feretos, 1964) with 0.6M KCl would result in the inclusion of significant myofibrillar Ca^{2+}-Mg^{2+}-ATPase activity (Cossins and Bowler, unpublished observations).

An additional complication to the interpretation outlined above is the protective effect of ions on both the (Na^+, K^+) ATPase and the Mg^{2+}-ATPase. This phenomenon suggests that an intracellular environment with high ionic content would cause a similar protection of these enzymes. Indeed, Gladwell (1973) has concluded from this experiment that the results of earlier experiments using muscle blocks, could not be applied uncritically to the events occurring <u>in vivo</u> during heat death.

In view of these problems, the *in vitro* inactivation at high temperature of an enzyme known to be associated with muscle membranes, and with a well-characterised function, was studied. The advantages of this FSR microsomal preparation have been outlined in Chapter 5, namely, its relatively high degree of purity, high specific activity and the possibility of studying both the ATPase and transport functions of the enzyme.

The experiments described in the Chapter were designed firstly to characterise the process of thermal inactivation of the Ca^{2+}-stimulated ATPase, and, secondly, to study its kinetics at certain temperatures to seek a correlation with the process of heat death of the whole animal. Finally, the inactivation of the Ca^{2+}-stimulated ATPase from cold and warm adapted crayfish were compared, to determine the effects of thermal acclimation upon this process.

MATERIALS AND METHODS

A. MATERIALS

1. Chemicals. Hepes (N-2-Hydroxyethylpiperazine N^{1}-2-ethanesulphonic acid) was obtained from the Sigma London Chemical Co. Ltd.
   The details and source of other reagents and chemicals have been described in Chapter 5.
2. Glassware. The source of all pipettes and glassware is given in Chapter 5, with the technique for washing them.
3. Animals. Freshwater crayfish (*Austropotamobius pallipes*) were caught and maintained in the laboratory as described in Chapter 5.

B. METHODS

1. Acclimation of Crayfish. Crayfish were acclimated at 4 ± 0.5°C or 25 ± 0.1°C as described in Chapters 2 and 5, for at least 21 and 7 days respectively (Bowler, 1963a). All animals were in the intermoult condition when sacrificed.

2. Extraction of Heavy Microsomal fraction from Crayfish Muscle. The abdominal flexor and extensor were rapidly dissected from the crayfish and the heavy (13,000 - 35,000g) microsomal isolated as described in Chapter 5.

During these experiments it became necessary to ensure that there was no contamination of the microsomal suspension by KCl from the previous 'salt extraction' step of the procedure. Therefore, the pellet formed by centrifugation of the microsomes in 0.6M KCl, was resuspended in the final suspension medium (details in the text) and centrifuged either at 35,000g for 60 minutes in an 'HS18' refrigerated centrifuge (M.S.E. Ltd.), or at 60,000g for 30 minutes in a 'SS40' ultracentrifuge (M.S.E. Ltd.), both at 4°C.

The pellet was finally resuspended in the final suspension medium by homogenisation and the preparation was used immediately or stored for up to 30 minutes on crushed ice.

3. Thermal Inactivation and Assay of Residual Enzyme Activity. The Ca^{2+} stimulated ATPase was inactivated at the desired temperature and the residual activity subsequently assayed using the following procedure.

0.5ml aliquots of the enzyme preparation were pipetted into centrifuged tubes which had been

equilibrated at the desired temperature. All preincubation temperatures were controlled to $\pm$ 0.1°C in large water baths using a 'Jumos' contact thermometer connected to a 500 watt immersion heater, through a hotwire vacuum relay switch. Temperatures were measured with a 0-50°C mercury thermometer (Gallenkamp Ltd.) which had previously been calibrated against a National Physical Laboratory certified thermometer.

After the desired period of preincubation, the tubes were rapidly transferred to a 25°C water bath, where, after 5 minutes equilbration, 1.5ml reaction medium containing Tris-ATP was added. The final concentration of the reaction media are detailed in Chapter 5, together with the techniques for determining both the Ca^{2+}-stimulated ATPase activity and the Mg^{2+}-dependent ATPase activity.

The enzyme reaction was stopped and the amount of inorganic phosphate liberated was estimated using the 'Lubrol' technique (see Chapter 5). Microsomal protein was estimated using the Folin-Ciocalteau reagent and BSA (fration V) as standard. (Lowry, Rosebrough, Farr and Randall (1951); see Chapter 5).

Specific activity was calculated as uM phosphate liberated.mg protein^{-1}.minute^{-1}. In most cases, however, enzyme activity is reported as a percentage of activity of an untreated preparation, which had been maintained on crushed ice until assayed.

4. Statistics and Theory.

(i) All statistical comparisons were performed using conventional techniques as described by Snedecor and Cochran (1967). Where appropriate, reference was made to the statistical tables of Fisher and Yates (1963).

(ii) Treatment of Inactivation Kinetics Data. If we assume first-order kinetics for the inactivation of an enzyme, then the rate of inactivation is related to the concentration of the active enzyme:

$-\frac{dA}{dt} \propto A$ where

$-dA/dt$ - rate of decrease in concentration of active form

A - initial concentration of active form

k - rate constant

$-\frac{dA}{dt} = kA$

Rearranging

$$-\frac{dA}{A} = k.dt \qquad \text{Equation - 8-1}$$

Integrating between two different concentrations of active enzyme Ao and At, which occurs over the time period t -

$$2.3 \log \frac{Ao}{At} = k.t. \qquad \text{Equation 8-2}$$

This may be rearranged to the linear form (y=mx+c)

$$+ \log At = \frac{-k.t.}{2.3} + \log Ao$$

Equation (8-3)

A plot of log At against t will yield a straight line where the slope is -k/2.303. In these studies it has been assumed that the concentration of the active enzyme is directly related to its enzyme activity. Thus the rate of inactivation was followed by assaying enzyme activity after various periods of preincubation. Residual activity was

expressed as a percentage of an untreated preparation (assayed in duplicate) and plotted as log % original activity against time.

The rate of inactivation was conveniently described by the half-time ('LD_{50}'), which was the time (minutes) for the enzyme activity of the preparation to decline to half its original activity. The LD_{50} is simply related to the rate constant, k, by the equation —

$$LD_{50} = {}^{0.693}/_{k}.$$

(iii) Activation Energy. The activation energy (Ea) for the inactivation process was calculated by converting the LD_{50} for each preincubation temperature to a rate measurement by taking reciprocals. An Arrhenius plot was constructed of ${}^{1}/LD_{50}$ against ${}^{1}/T^{o}K$, and the slope (A) of the line determined by regression analysis (Snedecor and Cochran, 1967). The Ea was computed from the equation

$$Ea = R \times 2.303 \times A \text{ Kcal.mole}^{-1}.$$

where R - gas constant = 1.978 cal. $mole^{-1}.{}^{o}K^{-1}$.

The activation energy is related to the activation enthalpy (ΔH^{*}) by the equation

$$\Delta H^{*} = Ea + R.T.$$

(iv) Entropy Change. The change in entropy associated with the inactivation process was calculated from the absolute rate theory equation (Dixon and Webb, 1964)

$$k = \frac{k_B T}{h'} e^{\frac{-\Delta H^{*}}{RT}} e^{+\frac{\Delta S^{*}}{R}}$$

where k_B - Boltzmann's Constant

h' - Planks' Constant

R - Gas constant

ΔH^* - Activation Enthalpy

ΔS^* - Activation Entropy

This was rearranged by taking natural logarithms to yield -

$$\ln k = \left(\ln \frac{k_B T}{h'}\right) - \frac{Ea}{RT} + \frac{\Delta S^*}{R}$$

and converted to $\log_{10}$ and rearranged -

$$\frac{\Delta S^*}{2.3R} = \log k - \log \frac{k_B T}{h'} + \frac{Ea}{RT}$$

RESULTS

It has been known for some time that a variety of factors may affect the thermostability of proteins. For example, Kunneman (1973) has found that the sensitivity of the lactate dehydrogenase from various fishes was markedly affected by the concentration of enzyme, presence of casein, serum albumen, $NADH^+$ and pyruvate. Several other authors have noted the effects of pH (Vessel and Yielding, 1966; Kono and Uyeda, 1971; Gatt, 1969; Kadlecova and Stepan, 1972; Bull and Breese, 1973), ionic environment (Coleman and Eley, 1962; Vessel and Yielding, 1966; Sudi, 1970; Wiseman and Williams, 1971; Theorell and Tatemoto, 1971) and other denaturing agents (Bull and Breese, 1973) upon protein denaturation.

It was important therefore to understand the process of heat inactivation of the Ca^{2+}-stimulated ATPase and the effect of different ionic conditions upon this process. The following experiments illustrate the effect of various ionic media and protein concentrations upon thermal inactivation of this enzyme.

A. The Effects of Various Ionic Media upon Thermal Inactivation

Figure 8-1a illustrates the effect of preincubation at 32°C upon the activity of a single Ca^{2+}-stimulated ATPase preparation, isolated from the muscle of variously acclimated

crayfish, which had been resuspended in different ionic media. The corresponding semi-log plots are presented in Figure 8-1b.

When resuspended in 10mM Imidazole-HCl, pH 7.1, or 10mM Hepes-KOH pH 7.1, the enzyme was rapidly inactivated at 32°C with a half-life (LD_{50}; time for the enzyme activity to be reduced to 50% of its original activity) of 4.5 minutes. The inactivation process followed pseudo-first order kinetics since the semilog plot in both cases was straight.

However, when resuspended in the presence of 100mM KCl, 10mM Imidazole-HCl, pH 7.1, the Ca^{2+}-stimulated ATPase activity was not inactivated by heat treatment but unexpectedly rose rapidly to approximately 180% of its original activity within 15 minutes of exposure to 32°C. The enzyme showed similar behaviour when resuspended in 10mM Imidazole-HCl, pH 7.1, but preincubated in the presence of the ionic media used for assay (i.e., 0.75mM $CaCl_2$, 0.75mM EGTA, 4.5mM $MgCl_2$, 150mM KCl in 44.1 Imidazole-HCl, pH 7.1).

When resuspended in neutralised deionised water only, the enzyme exhibited a reduced thermal sensitivity with a half-life of approximately 22 minutes. In other experiments it was shown that a considerable amount of K^+ was bound to the microsomal preparation, or from previous washes in the preparative procedure. Contaminating KCl may cause an increase in the thermal resistance of the enzyme. The inactivation process again conformed to pseudo-first order kinetics after an initial lag of 3-4 minutes. This may be caused by insufficient equilibration of the enzyme upon preincubation.

The thermal inactivation characteristics of a different microsomal preparation was assayed in the presence of 8.1 uM free calcium (0.5mM $CaCl_2$, 0.5mM EGTA) in 10mM Imidazole-HCl, pH 7.1 (Figure 8-1c). Again the preparation was inactivated

with pseudo-first order kinetics and a half-time of approximately 12 minutes.

It was concluded that low concentrations of $CaCl_2$ do not protect the Ca^{2+}-ATPase from thermal inactivation. High concentrations of KCl (i.e., 100mM) not only protects but markedly increases enzyme activity. Clearly the ionic composition of the resuspension medium has important consequences to the thermal stability of this membrane-bound enzyme.

B. The Effects of KCl upon Enzyme Thermostability

(i) Inactivation at high 'lethal' temperatures. A microsomal preparation isolated from 15°C-acclimated crayfish was resuspended either in 100, 10, 1 or 0.1 mM KCl, all in 10mM Imidazole-HCl, pH 7.1 and exposed to 32°C for varying period of time. The residual activity was immediately assayed at 25°C, and the results are presented in Figure 8-2a, b.

In the presence of 100mM KCl, the Ca^{2+}-stimulated ATPase showed the familiar increase in enzyme activity with preincubation at 32°C. However, in the presence of 10mM KCl the enzyme was inactivated with pseudo-first order kinetics and a half-life of 14 minutes. In the presence of 0.1 and 1.0mM KCl, the enzyme was inactivated more rapidly, with a half-life of 8 minutes.

It was apparent, therefore, that the enzyme was thermally inactivated at a maximal rate in the presence of low concentrations of KCl ($\ll$1mM), whilst 10mM KCl increases the enzymes thermal resistance and 100mM KCl protects and activates the enzyme activity.

It was not possible to reduce KCl concentration of the enzyme suspension below 0.1mM, since a sizable

contamination was introduced with the microsomal preparation as measured by atomic absorption spectrometry. This KCl was presumably bound to or within the microsomes since repeated washing in KCl-free media did not yield KCl-free microsomes.

In a separate experiment, a microsomal preparation resuspended in 10mM KCl, 10mM Imidazole-HCl, pH 7.1, showed a considerable increase in Ca^{2+} ATPase activity after preincubation at high 'lethal' temperatures (Figure 8-3a). The level of maximal activation was independent of preincubation temperature, although the higher the preincubation temperature, the more rapid was the activation. Interestingly, exposure of the enzyme preparation to 25°C also caused an increase in its activity, although this process occurred more slowly at this temperature.

In the experiment where the microsomes were first resuspended in 100mM KCl, and subsequently isolated from that medium by centrifugation at 35,000g for 60 minutes and resuspended in 10mM Imidazole-HCl, pH 7.1, the Ca^{2+}-stimulated ATPase of that preparation was equally sensitive to thermal inactivation as a preparation that was initially suspended in the imidazole medium. Evidently, suspension of the microsomes in a medium of high KCl concentration, does not in itself lead to activation or protection of enzyme activity. (Figure 8-3b)

(ii) Stability at 0,15 and 25°C

A number of enzymes have been shown to be affected by storage at low temperatures. For example, Kono and Uyeda (1971) have demonstrated that the phosphofructokinase from chicken liver is reversibly inactivated by storage at 0°C or 8°C, whereas storage at 25°C caused no

change in enzyme activity. The ATPase of beef heat mitochondria undergoes a fairly rapid inactivation at 0°C, but is stable at room temperature and even shows a slow increase in activity with storage (Pullman, Penefsky, Datta and Racker, 1960). On the other hand, the membrane-bound ATPase of human erythrocytes were found to be activated by freezing (Scharff and Vester Vestergaard-Bogind, 1966).

These studies encouraged a study of the stability of the microsomal Ca^{2+}-activated ATPase at different sublethal storage temperatures, partly in an attempt to explain the large variability in specific activity of different microsomal preparations (see Chapter 7).

Microsomal preparations from both 4°C and 25°C acclimated crayfish were resuspended in 10mM KCl, 10mM Imidazole-HCl, pH 7.1. Aliquots of each preparation were stored at 0°C, 15°C and 25°C, and subsequently assayed for residual activity. The results are illustrated in Figure 8-4.

Preincubation at 0°C led to a reduction in enzyme activity of approximately 12% per hour and 19% per hour in the preparations isolated from 4°C and 25°C acclimated crayfish, respectively. Preincubation at 15°C also led to a decrease in enzyme activity although it was only 11% and 13% per hour for the preparations isolated from 4°C and 25°C acclimated crayfish respectively.

Preincubation at 25°C caused a rapid increase in enzyme activity of 8 and 18% in the preparations isolated from 4°C and 25°C acclimated crayfish respectively. This was followed by a decline in activity at a rate of 12% and 16.5% per hour, respectively. It was concluded that the Ca^{2+}-stimulated ATPase is not cold labile.

Although the rate of decline in enzyme activity at the three storage temperatures were similar, the preparation isolated from 25°C acclimcated crayfish was somewhat more labile to storage than the corresponding preparation from 4°C acclimated crayfish. This is in agreement with the higher variability in the specific activity estimates of the former preparations described in Chapter 7. The cause of this phenomenon is not clear.

In a separate experiment the effect of different KCl concentrations upon the stability of the Ca^{2+}-ATPase at 0°C was studied. A microsomal preparation from the muscle of variously acclimated crayfish was resuspended in media containing 10, 5, 1, 0.5 and 0 mM KCl, all in 10mM Imidazole-HCl, pH 7.1. The preparation was stored on crushed ice for varying periods of time and the residual activity subsequently determined (Figure 8-5).

As before, the enzyme resuspended in 10mM KCl showed an initial increase in enzyme activity with preincubation at 0°C followed by a slow decline in enzyme activity. The microsomes resuspended at lower KCl concentrations, however, all showed a similar rate of decline of enzyme activity with preincubation at 0°C. There was no evidence of activation at these KCl concentrations.

It was concluded that the activation of enzyme activity on storage at all temperatures occurs only in the presence of high concentrations of KCl (i.e. $\geqslant$ 10mM KCl).

(iii) <u>Dependence of ATPase activity upon the concentration of microsomal protein.</u> During the course of these experiments, it became clear that the activity of any one microsomal Ca^{2+}-ATPase preparation was not a linear

function of the concentration of microsomal protein. This phenomenon is illustrated in Figure 8-6 , where the results of experiments on three preparations are shown. The microsomal protein concentration is given as a fraction of the original suspension to permit comparisons between preparations of different specific activity.

According to Dixon and Webb (1964), this form of enzyme concentration curve is caused by the presence of a dissociable activator or coenzyme. They state that as the concentration of the enzyme and activator/coenzyme increases, an increasing proportion of the enzyme molecules will be in the activated form, and an upward curvature of the graph will be obtained.

Preincubation of a microsomal preparation at 32°C for 20 minutes in the presence of 100mM KCl, 10mM Imidazole-HCl, pH 7.1 caused a marked increase in specific activity (see previous sections) of the preparation, and a change in the form of the enzyme concentration curve from the curvilinear form to a straight line which passed through the origin (Figure 8-6).

C. Irreversibility of Thermal Inactivation

Aliquots of a microsomal preparation isolated from the muscle of variously acclimated crayfish and resuspended in 5mM KCl, 10mM Imidazole-HCl, pH 7.1, was preincubated at 33°C for 5 minutes and stored at 0°C. The residual activity was subsequently assayed at 25°C after varying periods of storage at 0°C, to determine whether the activity of the enzyme recovered from thermal inactivation (Figure 8-7).

It is obvious that heat treatment resulted in a decreased enzyme activity at 25°C. Furthermore, the level of activity was not markedly affected by storage at 0°C. It was concluded, therefore, that thermal inactivation of this

enzyme under these conditions was an irreversible phenomenon. This indicates that the process is accompanied by a major change in the native structure of the enzyme, may even involve changes in the arrangement of disulphide bonds (Mahler and Cordes, 1971), and is in contrast to reversible enzyme inactivations which probably reflect less drastic alterations in molecular configuration. For example, the nucleotide pyrophosphatase of Proteus vulgaris may be completely inactivated by preincubation at 70°C for 10 minutes but may be completely reactivated by cooling to 37°C (Swartz, Kaplan and Lamborg, 1958; see also Swartz, Kaplan and Frech, 1956).

D. Effect of Protein Concentration upon Thermal Inactivation

(1) Concentration of microsomal protein. Kadelcova and Stepan (1972) have demonstrated that the thermostability of rat intestinal alkaline phosphatase is greatly influenced by the concentration of the tissue homogenate. Thus a 10% homogenate had a half-time at 56°C of over 20 minutes, whereas a 4% homogenate was 6 minutes and at 1% homogenate was 2 minutes. Vos and Boross (1972) have demonstrated a similar phenomenon using glyceraldehyde-3-phosphate. Kunneman (1973) and Wiseman and Williams (1971) have shown that heat-inactivation characteristics of enzymes are changed by inclusion of bovine serum albumin in the suspension medium.

Figure 8-8a, illustrates the thermal inactivation at 32°C of a microsomal Ca^{2+}-ATPase preparation, previously diluted twofold ($^{N}/2$) and fourfold ($^{N}/4$). The semilog plots for the N and $^{N}/4$ concentrations were almost identical with a half-life of approximately 4 minutes. The curve for the $^{N}/2$ dilution was similar to those described above for the first eight minutes,

but deviated considerably thereafter. In a separate experiment the inactivation curves at 33°C for N and $^{N}/_{2}$ dilutions were identical with a half-time of approximately 10 minutes and 9.3 minutes respectively (Figure 8-8b). The $^{N}/_{4}$ dilution was more thermosensitive with an LD_{50} of 7.1 minutes.

On balance, it was concluded that the rate of thermal inactivation of the Ca^{2+}-stimulated ATPase was not markedly affected by variations in the concentration of microsomal protein. However, in all subsequent experiments, the activity of each preparation was maintained at approximately this level in order to exclude this variable from the data.

(ii) Effect of BSA on enzyme stability. A microsomal Ca^{2+}-ATPase preparation was resuspended in media containing 5mM KCl, 10mM Imidazole-HCl, pH 7.1, with 0, 10, 50 and 100 ug bovine serum albumin/ml. The thermostability of each suspension was tested at 32°C in the usual manner and the results are presented in Figure 8-9a-d.

The half-time for inactivation of the enzyme in each medium was within one minute of the control suspension without BSA. It follows that variations of this magnitude in the total protein content of the microsomal preparations are not likely to have any effect upon the kinetics of thermal inactivation of this enzyme.

It is worth noting that Smith (1973a) has reported that succinoxidase in Tilapia mitochondria are largely protected from thermal inactivation by the incorporation of 1.5% BSA (w/v) into the suspension medium prior to preincubation. Indeed, 1-2% BSA is routinely used in the preparation of mitohondria and in the study of phospholipase activity on natural membranes since they

appear to chelate free fatty acids that otherwise might affect the enzymes. Wiseman and Williams (1971) have shown that horse liver alcohol dehydrogenase was protected from thermal denaturation by cysteine (800ug/ml) and BSA (30ug/ml).

E. Effect of Acclimation upon Thermal Inactivation Kinetics

The experiments described in the previous sections of this chapter demonstrate that the thermal inactivation of the Ca^{2+}-stimulated ATPase is a complex process, which depends upon the KCl concentration of the surrounding medium, at least. Experiments were therefore undertaken to compare the kinetics of thermal inactivation of this enzyme in preparations isolated from the abdominal muscle of 4°C and 25°C acclimated crayfish.

In all of the following experiments, the enzyme was resuspended in 5mM KCl, 10mM Imidazole-HCl, pH 7.1, because this concentration of KCl was small enough to allow inactivation to occur without inducing any activation phenomena, and large enough to make variations in the quantity of bound K^+ insignificant to the process of inactivation. In addition, the enzyme concentration of each preparation was adjusted to give approximately the same level of enzyme activity in each. The inactivation times were usually within 30 minutes, so that the effect of normal decay of enzyme activity with time was insignificant in relation to the thermal inactivation process.

(i) Ca^{2+}-stimulated ATPase activity. In each preparation the graph of log % residual activity against time was linear, indicating a first-order process with respect to time. The results of a typical experiment are illustrated in Figure 8-10a, where it is apparent that the Ca^{2+}-stimulated ATPase was very sensitive to treatment at 31-34°C. The higher the preincubation tempera-

ture, the more rapid is the inactivation process.

The LD_{50} (time for enzyme activity to be reduced by half) was estimated graphically for each preincubation temperature, and the change in LD_{50} with preincubation temperature was illustrated by a semilog plot of log LD_{50} against temperature, when a straight line was obtained (Figure 8-10b).

The results of experiments on several microsomal preparations from each acclimation group were averaged by averaging points taken from the log LD_{50}/temperature plots, since it was not possible to simply average the observed LD_{50} for each temperature because preincubation temperatures varied from experiment to experiment (see Table 8-1). There was some considerable variation in the slopes of the log LD_{50}/temperature plots particularly for those preparations isolated from the muscle of 25°C acclimated crayfish (see Table 8-1a). The high standard error for this acclimation group was caused mainly by one relatively thermoresistant preparation with an estimated LD_{50} of 46.4 minutes at 31.5°C, compared to the average of approximately 20 minutes. Nevertheless, preparations isolated from 25°C acclimated crayfish exhibited a greater mean LD_{50} at all preincubation temperatures (i.e., less temperature sensitive) than preparations isolated from 4°C acclimated crayfish (Figure 8-11 and Table 8-1b). For example, at 31°C the mean LD_{50} for former group of preparations was 27.9 ± 10.2 minutes, whereas for the latter it was 13.70 ± 1.36 minutes. This high variability in enzyme thermolability may be explained by the variability in heat resistance between animals of similar thermal histories (Bowler, 1963a; Gladwell, 1973).

These differences were statistically different only at the 34°C preincubation temperature (P = 0.02 - 0.05). It was therefore concluded that although preparations from 25°C acclimated crayfish showed a tendency towards increased thermal resistance, there was no overall difference between the two classes of preparation.

The activation energy (Ea) for the inactivation step between 31°C and 34°C was estimated by regression analysis of Arrhenius plots as described in the 'Methods', and the results for each preparation are given in Table 8-2. The Ea's for each acclimation group were averaged and compared statistically (Table 8-2). The mean Ea for preparations isolated from 4°C acclimated crayfish was 92.29 ± 14.63 Kcal.mole^{-1} (386.42 ± 61.24 KJ.mole^{-1}). and 100.58 ± 10.47 Kcal.mole^{-1} (421.14 ± 43.86 KJ.mole^{-1}) for the preparations isolated from 25°C acclimated crayfish. This difference was not statistically significant (P = 0.6 - 0.7).

(ii) Mg^{2+} dependent activity. The thermostability of the Mg^{2+}-ATPase activity of a heavy microsomal preparation was tested at temperatures between 31 and 33.5°C (see Figure 8-12). It is clear that there is no reduction in enzyme activity with preincubation at these temperatures.

F. Effect of Heat Death of the Whole Animal upon the Ca^{2+}-ATPase activity of the Heavy Microsomal Fraction of Muscle

The protective effects of high KCl concentrations upon the *in vitro* inactivation of the microsomal Ca^{2+}-ATPase suggests that this enzyme would not be inactivated *in vivo*, since the bulk sarcoplasmic K^+ concentration is approximately 120mM (Gladwell, 1973).

In order to test this hypothesis, three crayfish of roughly equal size were selected at random from a population and incubated at 15°C (A), 35°C (B) and 55°C (C) for 10 minutes respectively. The animals incubated at 35°C and 55°C were heat dead by the criteria of Bowler (1963a) and Gladwell (1973). The abdominal muscle from each animal was subsequently dissected out and the heavy microsomal fraction of each muscle prepared in the normal manner.

The final pellets for the preparations isolated from crayfish incubated at 15°C and 35°C appeared a normal straw-colour, but that from the 55°C treated crayfish muscle was a bright scarlet. This was probably caused by contamination by astaxanthin, a dietary pigment that is incorporated into crayfish tissues as an xanthoprotein.

The specific activity of each microsomal preparation was assayed at 25°C (Table 8-3). The preparations from both control (15°C) and 35°C heat dead crayfish showed vigorous Ca^{2+}-stimulated and Mg^{2+}-dependent ATPase activity, but the preparation isolated from 55°C treated crayfish was completely inactive.

It is apparent from this experiment that heat death of the whole animal at 35°C is not accompanied by an irreversible inactivation of the Ca^{2+}-ATPase. This temperature causes total inactivation of this enzyme *in vitro* within 5 minutes.

DISCUSSION

The temperature range in which the freshwater crayfish undergoes heat death is very similar to the temperature range over which the Ca^{2+}-stimulated ATPase is inactivated. This correspondence is made more dramatic by consideration of the LD_{50}/temperature curve for the thermal inactivation of a soluble crayfish enzyme,

muscle pyruvate kinase, which occurs between 55°C and 65°C (see Figure 8-13; Cossins, unpublished observations). The LD_{50} for the inactivation of the Ca^{2+}-ATPase isolated from both 4°C and 25°C acclimated crayfish was more rapid at the same temperature than the LD_{50} for heat death of the whole animal (Figure 8-13). However, one should not necessarily expect a precise correlation between these parameters, since the magnitude of the LD_{50} depends upon the arbitrary criterion of heat death. Thus Bowler (1963a) described death as occurring when all movements of the animal had ceased, whereas Gladwell (1973) used cessation of scaphognathite beat as his criterion of heat death.

The Ca^{2+}-stimulated ATPase of crayfish FSR and the MgATPase of crayfish muscle microsomes (Gladwell, 1973; Bowler and Duncan, 1967; Bowler, Duncan, Gladwell and Davison, 1963) therefore conform to the criterion implied by Read (1967) that there may be some relationship between the heat death of an intact animal and the thermostability of a constituent protein, if the temperatures at which both processes fail coincide closely.

However, it is considered unlikely on two counts that the Ca^{2+}-stimulated ATPase is inactivated <u>in vivo</u> during heat death. Firstly, it has been shown that the Ca^{2+}-stimulated ATPase activity of a microsomal preparation isolated from the muscle of a heat dead crayfish appeared to be fully functional. Secondly, the presence of a high ionic media <u>in vitro</u> protected the enzyme from thermal inactivation. This being the case, it is difficult to decide upon the physiological significance of the inactivation phenomena studied <u>in vitro</u>.

The fact that both the Ca^{2+}-stimulated ATPase and the Mg^{2+}-dependent ATPase and the (Na^+, K^+) ATPase of the light muscle microsomes were inactivated over the same temperature range (30-35°C, Figure 8-13) and with a similar activation energy (Table 8-4), suggests that there may be some common factor

involved in the inactivation process. This common factor may be their solvent environment, the hydrophobic core if the membrane. It is suggested that under certain conditions of low ionic strength and elevated temperature, the molecular motion of the alkyl chains of the membrane phospholipids becomes so great, that membrane integrity is disrupted and the constituent enzymes are exposed to various stresses, which may result in a reorganisation of their fragile three-dimensional configuration into an inactive form. The limited temperature range of this phenomenon suggests that it is a co-operative effect (Mahler and Cordes, 1971), and may be caused either by a direct effect of the molecular mobility of the hydrocarbon environment upon enzyme structure, or by the intrusion to varying degrees of an aqueous media into the hydrocarbon core, thereby disrupting the thermodynamic forces which dictate the higher order configuration of the protein molecule.

This hypothesis would explain the increased conductivity of the sarcolemma at heat death temperatures (Gladwell, 1973) and the consequent loss of K^+ and gain of Na^+ by crayfish muscle during heat death (Gladwell, 1973) since various studies have demonstrated that the permeability of model membrane systems to non-electrolytes increases markedly at elevated temperatures (see Chapter 3). Gladwell (1973) has also demonstrated that the increase in membrane conductance, and decrease in the membrane resting potential, that occur during heat death are reversible if the preparation is returned to normal temperatures.

A prediction of this hypothesis is that if enzyme inactivation is a consequence of the breakdown of membrane integrity, then membranes from warm-adapted animals which contain a smaller proportion of unsaturated fatty acids (Chapter 3) should be more stable at any single temperature than membranes from cold-acclimated crayfish. This phenomenon would be manifest as a

greater resistance of membrane-bound enzymes to lethal heat stress. The absence of any acclimation effect upon the kinetics of thermal inactivation of the Ca^{2+}-stimulated ATPase of the crayfish FSR was disappointing, and was in contrast to the results of Gladwell (1973; see also Bowler, Duncan, Gladwell and Davison, 1973) using the Mg^{2+} ATPase of a crayfish muscle microsomal preparation. In this context, it is interesting to note that Tume, Newbold and Horgan (1973) failed to observe changes in various ATPase and Ca^{2+}-sequestration properties in FSR preparations from rats which had been fed fat-supplemented diets, even though the fatty acid composition of the sarcoplasmic reticulum was markedly affected. The Ca^{2+}-ATPase of the sarcoplmasic reticulum may, therefore, represent an enzyme that is more independent of its lipid environment than other membrane-bound enzymes. As such it remains a major obstacle to the hypothesis. However, it is not certain that the fatty acid composition of those phospholipids associated with either the sarcoplasmic reticulum or more specifically with the Ca^{2+}-stimulated ATPase, is modified by acclimation to a different environmental temperature, to the same extent as the total lipid fraction of crayfish muscle. Alternatively, the inactivation of the Ca^{2+}-stimulated ATPase may not be influenced by the nature of its hydrophobic environment. As noted earlier, the ionic conditions during thermal inactivation were quite different to those believed to exist <u>in vivo</u>. Study of thermal inactivation kinetics at 100mM KCl, <u>after</u> the activation phase may demonstrate acclimation-dependent differences.

The time course of the thermal inactivation of the microsomal Ca^{2+}-stimulated ATPase was adequately described as a first-order process, where the rate of inactivation of the enzyme was directly proportional to the concentration of active enzyme. There was apparently no deviation from first-order kinetics, even when the

process was near to completion, suggesting that the process was essentially irreversible and quite distinct from the more complex thermal inactivation characteristics of the(Na^+-K^+)-dependent ATPase and its subunits from pig brain, as described by Atkinson, Gatenby and Lowe (1971). However, Brandt (1967) maintains that most protein denaturation reactions are reversible, provided the solvent conditions are correct and carefully controlled. First order thermal inactivation kinetics have been described for a number of soluble enzymes, such as the nitrate reductase from a Halobacterium salinarium (Marques and Brodie, 1973); Alkaline phosphatase from rat tissues(Kadlecova and Stepan, 1972); erythrocyte acetylcholinesterase (Coleman and Eley, 1963); Horse liver alcohol dehydrogenase (Theorell and Tatemoto, 1971) and Rat plasma corticosterone-binding protein (Hocman and Alexandrova, 1972), and membrane bound enzymes such as the Mg^{2+}-ATPase of crayfish sarcolemma (Gladwell, 1973) and the microsomal (Na^+, K^+)-ATPase of rat brain (Kirschmann, Levy and De Vries, 1973).

In Table 8-4, the derived thermodynamic parameters ΔH^* and ΔS^* for the inactivation of the Ca^{2+}-stimulated ATPase of crayfish FSR, and several other soluble and membrane-bound enzymes are compared, together with those parameters for heat death of the whole animal. The entropy and enthalpy changes during thermal inactivation of proteins and the heat death of complex animals are exceedingly large (see Evans and Bowler, 1973, for ΔS^* and ΔH^* for heat death and ageing in a number of poikilotherms); the ΔH^* and ΔS^* for most chemical and enzymatic reations are 5-25 Kcal. $Mole^{-1}$ (26.6-103.1 KJ $mole^{-1}$) and +10 to -40 entropy units/mole, respectively. This comparison emphasises in a dramatic fashion the degree of structural reorganisation that occurs during both enzyme inactivation and heat death. It is worth noting that ΔS^* and ΔH^* may vary with temperature. For example, Tanford (1970) has shown that ΔS^* for the denaturation of ribonuclease at pH 2.5,

and 0°C is 31 cal.deg^{-1}.mole^{-1}., at 25°C is 155 cal.deg^{-1}.mole^{-1}. and at 50°C is 340 cal.deg^{-1}.mole^{-1}. The relatively low value of ΔH^* for the inactivation of the (Na^+, K^+) ATPase of crayfish sarcolemma (Table 8-4) may be increased at higher temperatures in conformity with the ΔH^* values of 100-300 Kcal.mole (418.7-1461.0 KJ mole^{-1}.) that is characteristic of inactivation of many other proteins.

ΔH^* and ΔS^* for the inactivation of the Ca^{2+}-stimulated ATPase were somewhat lower than the corresponding values for heat death of the whole crayfish (Table 8-4). It is evident from this comparison that the inactivation of the Ca^{2+}-stimulated ATPase requires less thermal energy and undergoes a smaller entropy change than does the process of heat death. Indeed, one might expect these parameters for the latter phenomenon to be the sum of several complex events, each having a particular enthalpy requirement. It is interesting that these thermodynamic quantities for inactivation of the Ca^{2+}-stimulated ATPase and pyruvate kinase fall on the 'compensation' line of Rosenberg, Kemeny, Switzer and Hamilton (1971). However, the interpretation of this phenomenon is controversial (Banks, Danjanovic and Vernor, 1972; Evans and Bowler, 1973).

The protection of enzymes and proteins from thermal inactivation by salts is a well known phenomenon (Marquez and Brodie, 1973; Kirschmann, Levy and De Vries, 1973; Coleman and Eley, 1963). Solaro, Gertz and Briggs (1972) have reported that sucrose could protect the Ca^{2+}-uptake activity of canine skeletal muscle FSR from the inhibiting effects of exposure to 37°C.

The increase in enzyme activity by exposure to elevated temperatures has also been observed by Gladwell (1973) in the (Na^+, K^+) ATPase of crayfish muscle in the presence of NaCl, KCl and $MgCl_2$, and by Bowler and Duncan (1973) in the same preparation. Vianna, Bhatnagar and Gergely (1971) have described an increased Ca^{2+}-ATPase activity of a rabbit FSR preparation preincubated at

room temperature in the prescence of various anions and Hartshorne, Barns, Parker and Fuchs (1972) have shown that preincubation of actomyosin preparations to temperatures between 44°C and 54°C resulted in an enhanced ATPase activity, both in the presence of calcium and magnesium, and in the presence of magnesium alone. There is a similar increase in the ATPase activity of beef heart mitchondria during preincubation at 30°C, but a marked inactivation at 0°C (Pullman, Penefsky, Diatta and Racker, 1960; see also Aithal, Kalra and Brodie, 1974). On the other hand, the presence of Ca^{2+} or Mg^{2+} has been shown to increase the thermal sensitivity of mammalian erythrocyte Ca^{2+}-Mg^{2+}-ATPase (Bond, 1972).

It should be remembered that the protection and activation processes may be phenomenologically distinct processes, since they can occur either sequentially in the same preparation (i.e., activation followed by inhibition) or separately (i.e., inactivation with no activation phase). Characteristically, these processes require high concentrations of KCl and exposure to elevated temperatures for a period of time. Activation of the Ca^{2+}-stimulated ATPase appeared to be associated with a change in the shape of the enzyme concentration/activity curve. In the inactivated state the line is curving upwards, whereas after activation enzyme activity is directly proportional to its concentration. It may be that treatment at high temperature with excess KCl causes maximal activation by some activator. Bond and Clough (1973) have demonstrated such a soluble protein activator in the (Mg^{2+}, Ca^{2+}) dependent ATPase of human erythrocyte membranes.

Certain soluble enzymes are converted from a completely inactive form into an active form by heating at 60-100°C, or by treatment with urea, acetone or detergents (Swartz, Kaplan and French 1958). In these cases activation appears to be related to the inactivation of a naturally occurring inhibitor of the enzyme. A necessary corrollary of this phenomenon is that the

enzyme be heat-stable. Such a situation is unlikely to explain the activation of the Ca^{2+}-stimulated ATPase of crayfish FSR in view of the low thermal resistance of the enzyme and the possibility of enzyme activation discussed previously.

The mechanism of these effects is unknown. It may represent a direct effect either on the enzyme or the membrane phospholipids, or on the interactions between these two processes. Marquez and Brodie (1973) have suggested that protection of enzymes from halophilic bacteria by high concentrations of salt, is due to the 'shielding' of negative electrical charges on the enzyme, or perhaps to increased hydrophobic interactions within the protein molecule, both of which result in a tighter, more stable protein configuration. Brandt (1967) has emphasised that the configuration state of a protein molecule is thermodynamically controlled. He described the types of forces involved in the formation of higher-order protein structures and shows that the solvent environment has important effects both on the intramolecular forces and the enzyme structure.

On the other hand, inorganic ions do have marked effects upon the physical state of membrane lipids. Calcium (for example) interacts strongly with the headgroups of acidic phospholipids, resulting in an increase in the surface potential and a decrease in surface pressure of a monomolecular layer of phospholipids at the air-water interface (Papahadjopoulos, 1968). Ohnishi and Ito (1973, 1974) have shown that calcium can induce phase separation in bilayers composed of phosphatidyl choline and phosphatidyl serine. This results in a reduction in the lateral diffusion of membrane lipids, and also the molecular motion of their hydrocarbon chains (Chapman, Urbina and Keogh, 1974), both of which result in a diminution of the kinetic stress upon the protein molecule. Potassium has not been demonstrated to possess these extensive membrane-active properties, although they may reduce the repulsive

forces between adjacent phospholipids by salt formation with their phosphate moeity. The adsorption of counter ion onto the membranes has an enormous influence on the 'zeta' potential of the membrane (i.e., the electrical potential at the plane of shear, see Dawson, 1968). Often univalent counter ions can, if present in sufficiently high concentrations, reduce the zeta potential to negligible proportions. This effect will have great effects upon the state of bound water, and the permeability characteristics of the membrane, as well as upon the state of the hydrocarbon core itself.

Table 8-1a: The Estimated LD_{50} for Ca^{2+}-stimulated ATPase Activity at Different Preincubation Temperatures for Microsomal Preparations isolated from 4°C and 25°C acclimated crayfish

Methods: Values for LD_{50} at 31, 32, 33 and 34°C were estimated graphically from the log LD_{50}/temperature plot for each preparation.

Table 8-1b: The Average LD_{50} at different Preincubation Temperatures for the Thermal Inactivation of the Microsomal Ca^{2+}-stimulated ATPase from 4°C acclimated and 25°C acclimated crayfish

Methods: For details, see Figure 8-11

Table 8-1a

Acclimation Temperature	Preparation Number	Preincubation Temperature °C			
		31	32	33	34
4	1	14.1	8.2	4.6	2.7
	2	9.8	6.8	4.7	3.2
	3	16.0	8.4	4.5	2.4
	4	14.9	8.7	5.1	3.0
25	1	11.8	7.2	4.4	2.70
	2	12.7	8.4	5.5	3.6
	3	19.2	11.5	6.8	4.0
	4	29.0	15.9	8.6	4.7
	5	67.0	29.5	13.2	5.8

Table 8-1b

Preincubation Temp.	4°C Acclimated Crayfish Mean $LD_{50} \pm$ SEM (n = 4)	25°C Acclimated Crayfish Mean $LD_{50} \pm$ SEM (n = 5)	t	P	Significance
31	13.70 ± 1.36	27.94 ± 10.24	1.38	0.2-0.4	N.S.
32	8.03 ± 0.42	14.50 ± 4.04	1.656	0.2-0.1	N.S.
33	4.73 ± 0.13	7.70 ± 1.54	1.916	0.05-0.1	N.S.
34	2.82 ± 0.18	4.16 ± 0.52	2.427	0.02-0.05	S.

Table 8-2: The Regression Data for Arrhenius plots of Thermal Inactivation of the Ca^{2+}-ATPase and the Activation Energy of this process in Microsomal Preparations isolated from 4°C and 25°C acclimated crayfish

Methods: See 'Materials and Methods'

Table 8-2

Preparation Number	Acclimation Temperature (^{o}C)	Slope b	Ea (Kcals.mole^{-1})	Ea (KJ mole^{-1})	r	Mean Ea (Kcal.mole^{-1})	SEM	t_*	P
1		-21.12	96.65	404.68	-0.9974				
2		-23.61	108.04	452.36	-0.9979	-100.58	10.47		
3	25	-15.13	69.24	289.89	-0.9787				
4		-29.26	133.90	560.62	-0.9903			0.461	0.6-0.7
5		-20.79	95.14	398.34	-0.9986				N.S.
6		-24.56	112.4	470.57	-0.9761	92.29	14.63		
7	4	-13.95	63.84	267.28	-0.9955				
8		-21.99	100.63	421.33	-0.9725				

Table 8-3: The Effect of Treatment of the whole animal at Lethal Temperatures upon the activity of microsomal Ca^{2+}-stimulated ATPase subsequently extracted

Treatment	15°C for 10 minutes A	35°C for 10 minutes B	55°C for 10 minutes C
Ca^{2+}-stimulated ATPase Activity *	0.727	0.744	0
Mg^{2+}-dependent ATPase Activity *	0.083	0.051	0
Protein content of suspension ug/ml	157.5	151.2	66.5

* uM Pi liberated. mg protein^{-1}. minute^{-1}.

Table 8-4: The values of ΔS^* and ΔH^* for the inactivation of several membrane-bound enzymes and soluble enzymes and the process of heat death in the freshwater crayfish

Enzyme	ΔH^*	ΔS^*	Reference
Ribonuclease Chymotrypsinogen Myoglobin	57 39 42	185 105 95	Mahler and Cordes, 1971
Crayfish FSR Ca^{2+} ATPase	98.1	245.4	Present work
Crayfish Pyruvate Kinase	71.4	141.4	Cossins (unpublished)
Crayfish sarcolemma Mg^{2+} ATPase **	103-114	-	Gladwell 1973, Bowler and Duncan 1967
Crayfish sarcolemma (Na^+, K^+) ATPase **	40-48	-	Gladwell 1973, Bowler and Duncan 1967
Crayfish, heat death	137.0	377.0	Evans and Bowler, 1973

** data includes values for differently acclimated crayfish.

- data not available.

Figure 8-1: The effect of different ionic media upon the thermal inactivation characteristics of the microsomal Ca^{2+}-ATPase

Methods: a, b - A microsomal preparation was resuspended in various ionic media and aliquots of each were preincubated at 35°C. The activity remaining was assayed at 25°C. Values are expressed as a % of the activity of an untreated aliquot and plotted in linear (a) and semi-log form (b).

c, d - A separate microsomal preparation resuspended in 0.5 mM $CaCl_2$, 0.5 mM EGTA in 10 mM Imidazole-HCl pH 7.1, and treated as above. Values were plotted in linear (c) and semi-log (b) form.

Legend: Abscissa: Period of Preincubation at 35°C (minutes).

Ordinate: % Residual Activity.

● Neutralised Deionised water

▲ 10 mM Hepes-KOH, pH 7.1

△ 10 mM Imidazole-HCl, pH 7.1

▣ 100 mM KCl, 10 mM Imidazole pH 7.1

○ Assay Medium

□ 0.5 mM $CaCl_2$, 0.5 mM EGTA, 10 mM Imidazole-HCl pH 7.1

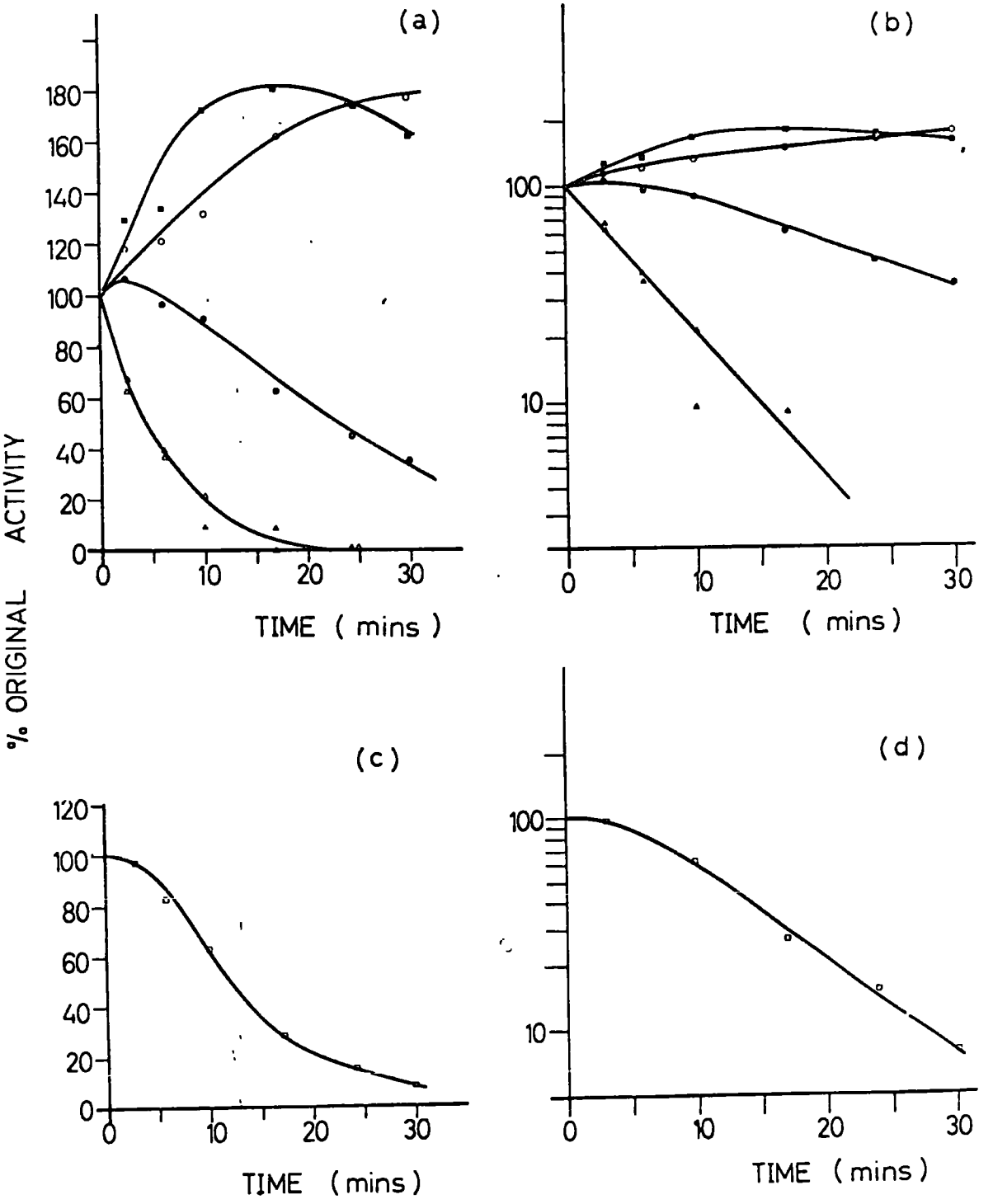

(a)
(b)
(c)
(d)
% ORIGINAL ACTIVITY
TIME (mins)
TIME (mins)
TIME (mins)
TIME (mins)
0
10
20
30
40
60
80
100
120
140
160
180

Figure 8-2: The effect of different KCl concentrations upon the thermal inactivation characteristics of the microsomal Ca^{2+}-ATPase

Methods: A microsomal preparation was resuspended in different KCl concentrations, and the thermal inactivation kinetics at 32°C determined as described in the 'Methods'. Values were plotted in linear (a) and semi-log forms (b).

Legend: Ordinate: % Residual Activity

Abscissa: Preincubation Time at 32°C (minutes)

- ● 100 mM KCl, 10 mM Imidazole-HCl pH 7.1
- □ 10 mM KCl, 10 mM Imidazole-HCl pH 7.1
- ▲ 1.0 mM KCl, 10 mM Imidazole-HCl pH 7.1
- △ 0.1 mM KCl, 10 mM Imidazole-HCl pH 7.1

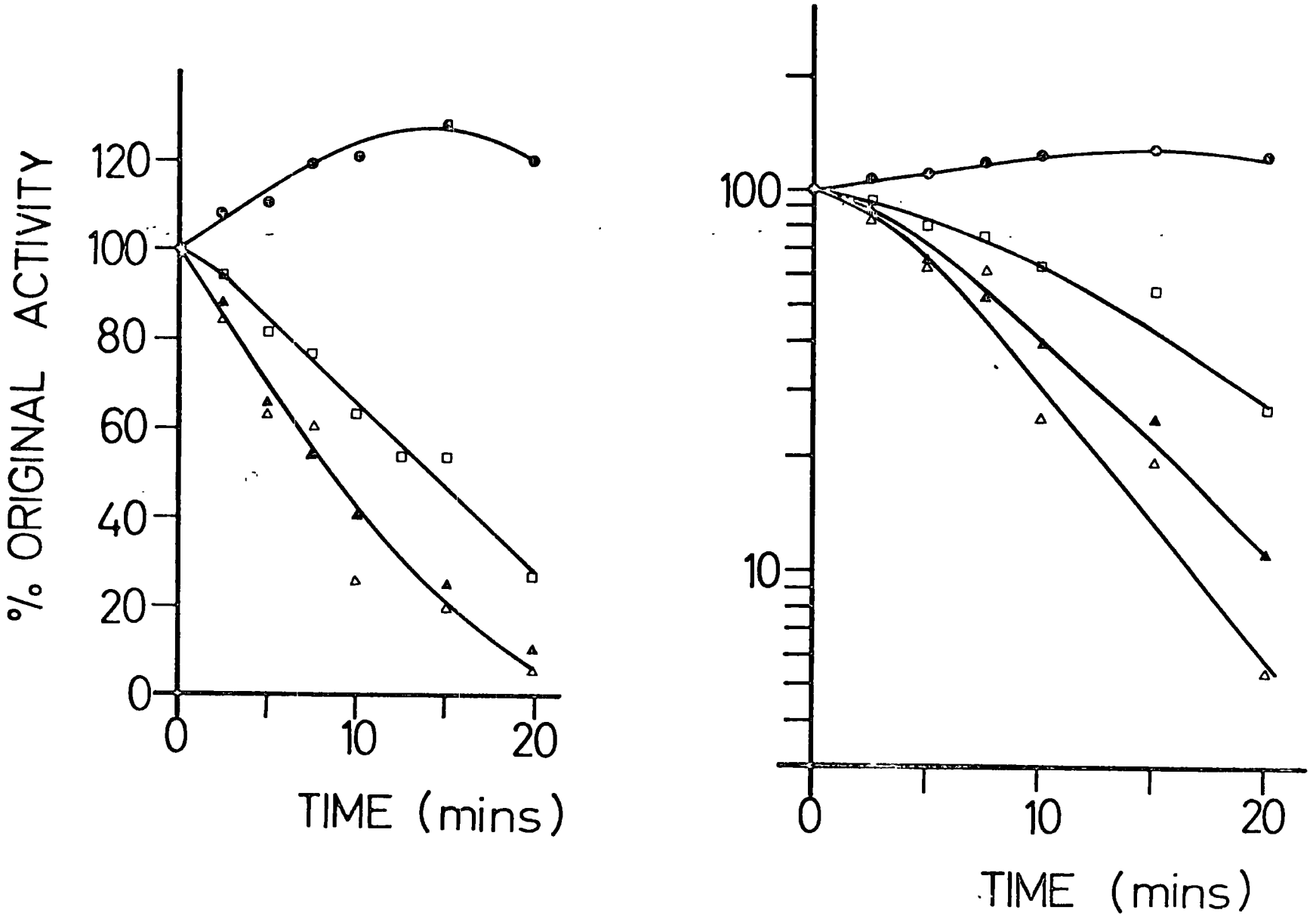

% ORIGINAL ACTIVITY
120
100
80
60
40
20
0
0
10
20
TIME (mins)
100
10
0
10
20
TIME (mins)

Figure 8-3: The effect of preincubation at high temperature in the presence of 100 mM KCl

Methods: a) A microsomal preparation was resuspended in 100 mM KCl, 10 mM Imidazole-HCl, pH 7.1 and preincubated at 31-34°C for varying periods of time. Residual activity was assayed at 25°C.

b) A separate aliquot was centrifuged at 35,000 g for 60 minutes, and the pellet was resuspended in 10 mM Hepes-KOH, pH 7.1. The thermal inactivation of this preparation was tested at 32°C.

Legend: Ordinate: % Residual Activity

Abscissa: Preincubation Time (minutes)

■ Preincubation Temperature - 31.5°C

○ 32.5°C

△ 34°C

□ Resuspended preparation, preincubated at 32°C.

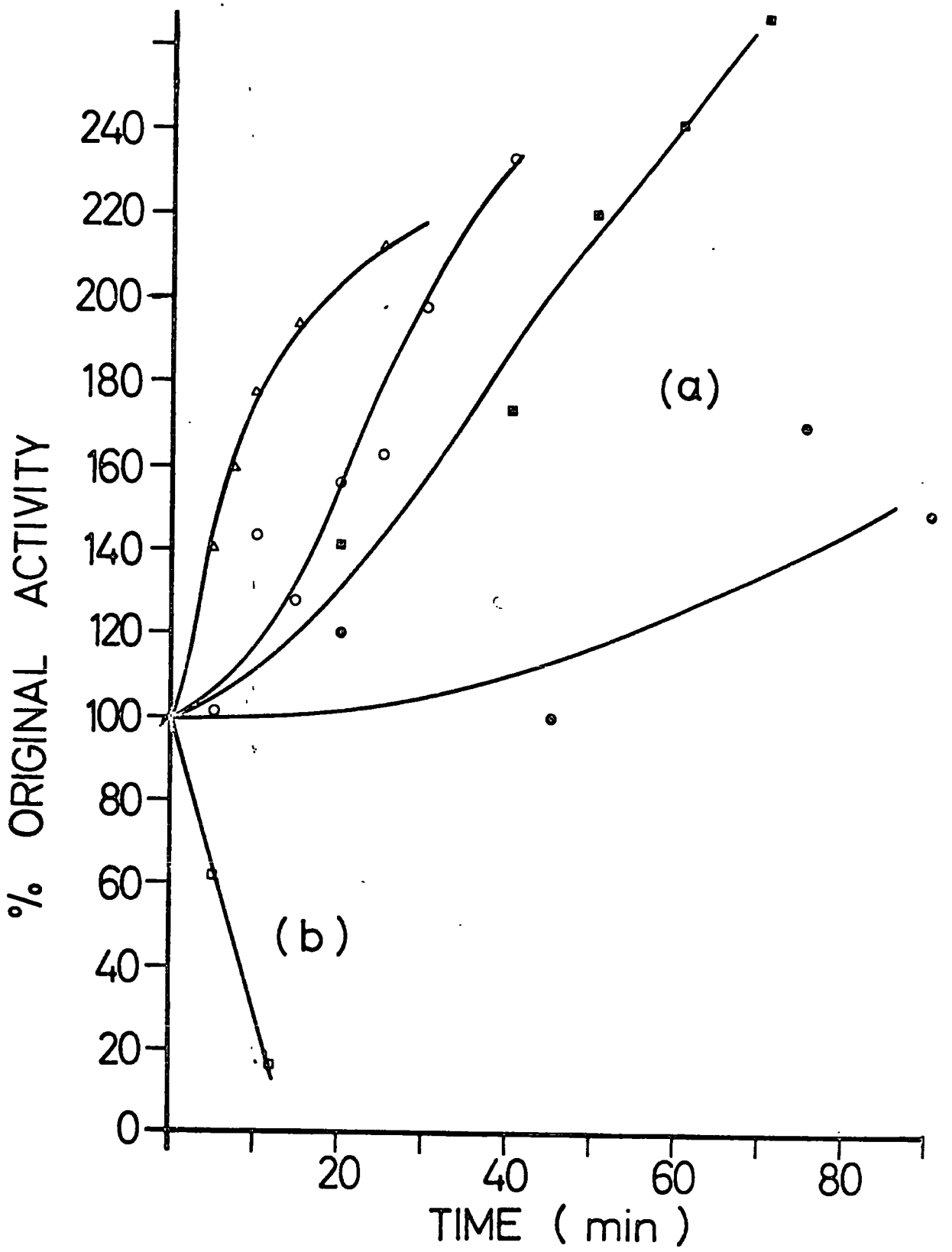

% ORIGINAL ACTIVITY
240
220
200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
TIME (min)
(a)
(b)

Figure 8-4: Stability of Ca^{2+}-ATPase at 0, 15 and 25°C

Methods: Microsomal preparations from 4°C (a) and (b) 25°C acclimated crayfish were stored at 0, 15 or 25°C. At various periods of times, 0.5 ml aliquots were removed and immediately assayed at 25°C for residual activity.

Legend: Ordinate: % Residual Activity

Abscissa: Storage Time (minutes)

Preparation from 4°C acclimated crayfish:

Storage Temperature 0°C ●

15°C ▼

25°C ■

Preparation from 25°C acclimated crayfish:

Storage Temperature 0°C ●

15°C ▼

25°C ■

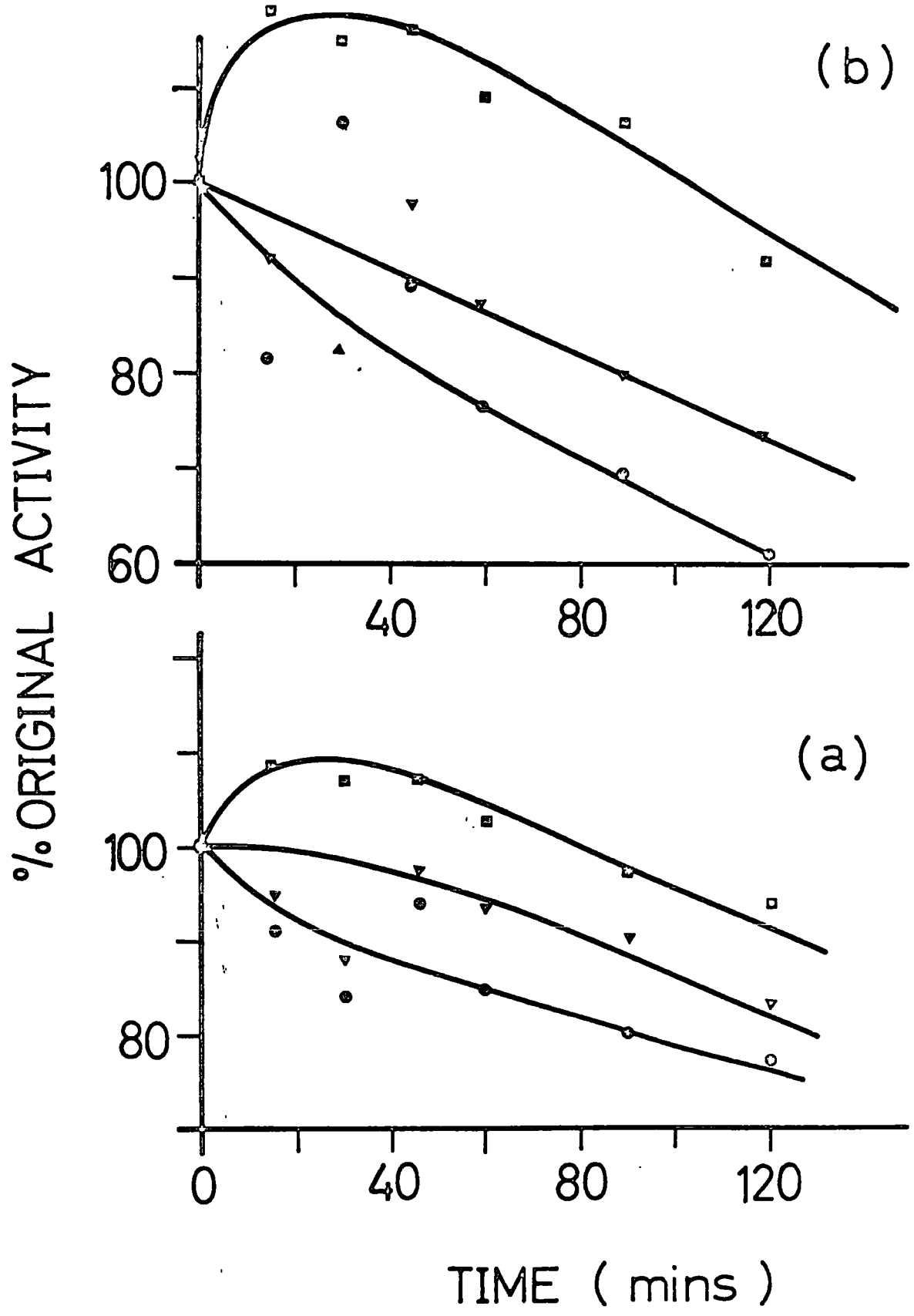

(b)
100
80
60
40
80
120
% ORIGINAL ACTIVITY
(a)
100
80
0
40
80
120
TIME (mins)

Figure 8-5: Effect of different KCl media upon the stability of the microsomal Ca^{2+}-ATPase at 0°C

Method: A microsomal preparation was resuspended in media of different KCl concentration, but all containing 10 mM Imidazole-HCl, pH 7.1. The effect of storage at 0°C for 1 hour was determined as described for Figure 8-4.

Legend: Ordinate: % Residual Activity

Abscissa: Preincubation Time at 0°C (minutes)

● 10 mM KCl

△ 5 mM KCl

▲ 1 mM KCl

□ 0.1 mM KCl

○ 0 mM KCl

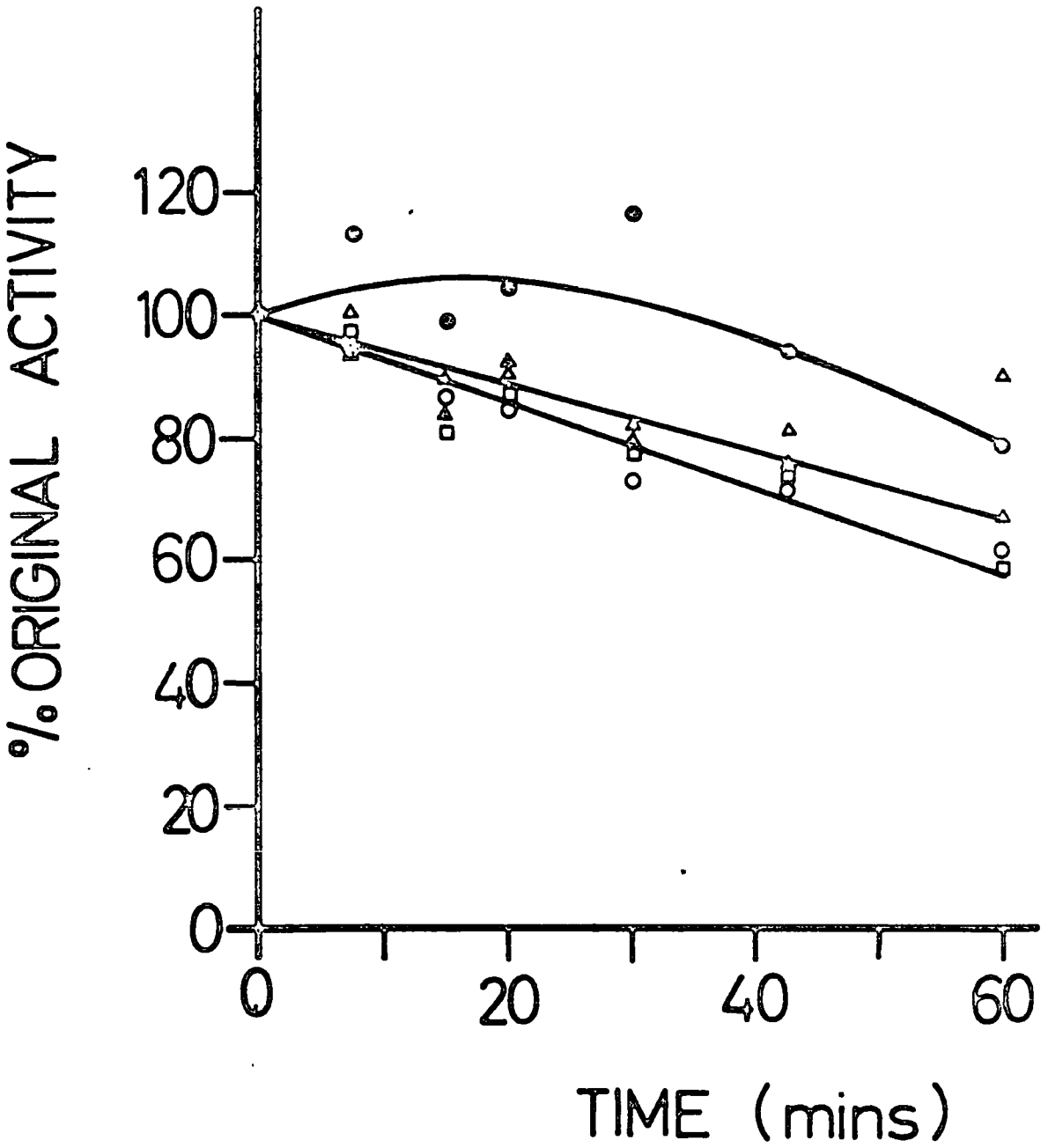

% ORIGINAL ACTIVITY
120
100
80
60
40
20
0
0
20
40
60
TIME (mins)

Figure 8-6: The effect of enzyme concentration upon Ca^{2+}-ATPase activity for normal and heat treated preparations

Methods: (a) Microsomal preparations were diluted two-fold, three-fold and four-fold and the enzyme activity of each diluant determined at 25°C (b). A separate preparation was treated at 32°C for 20 minutes before dilution and assay at 25°C.

Legend: Ordinate: % Activity of Original Preparation

Abscissa: Relative Protein Concentration

Microsomes in 100 mM KCl

Microsomes in 10 mM KCl

Microsomes in 100 mM KCl but heat treated prior to assay (b).

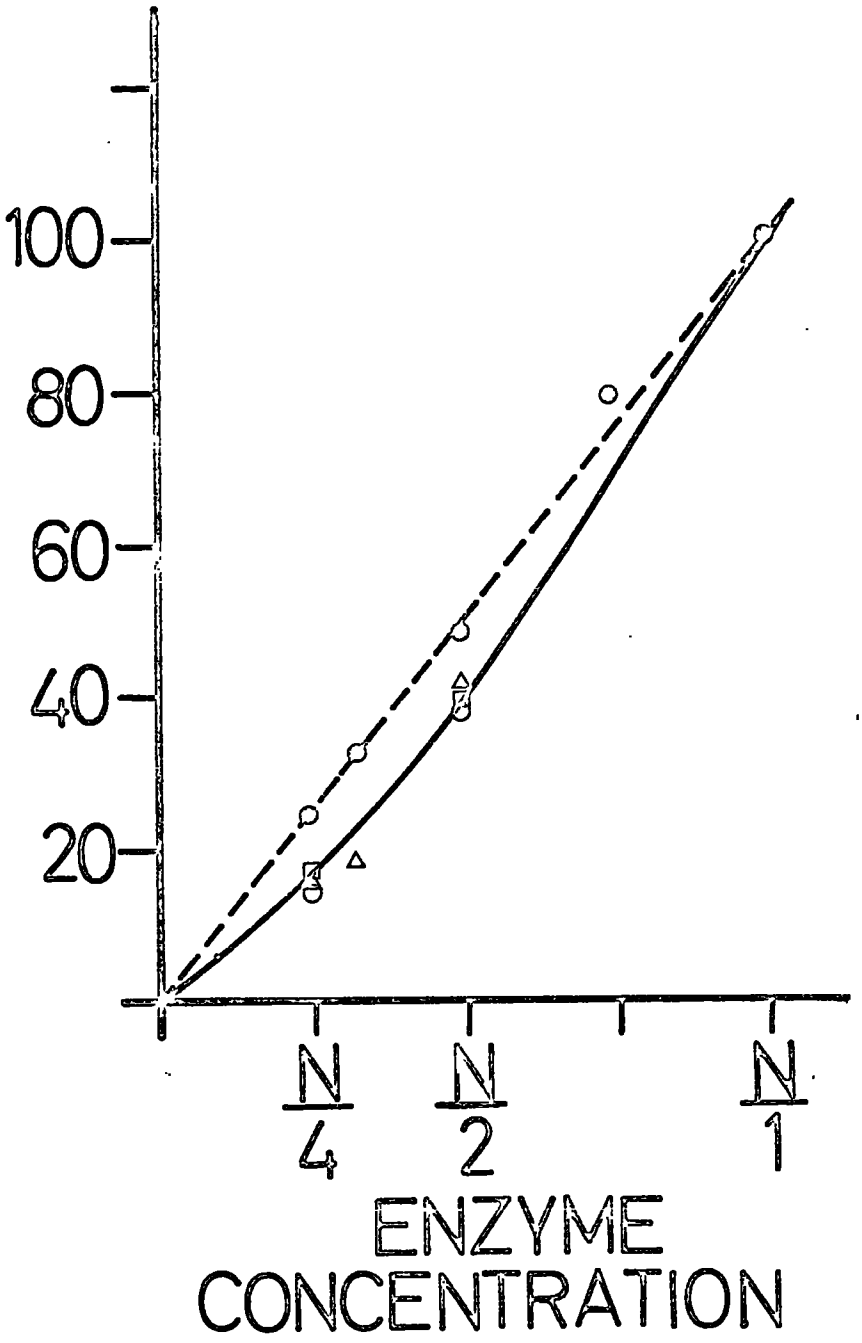

100
80
60
40
20
N/4
N/2
N/1
ENZYME CONCENTRATION

Figure 8-7: Effect of storage at 0°C upon the Ca^{2+}-ATPase activity of a microsomal preparation that had been partially inactivated by high temperature

Method: A microsomal preparation was treated at 33°C for 5 minutes and transferred to ice. At various intervals aliquots of the microsomal preparation were assayed for Ca^{2+}-ATPase activity at 25°C. Values were calculated as % en yme activity prior to heat treatment

Legend: Ordinate: % Original Activity

Abscissa: Storage Time at 0°C

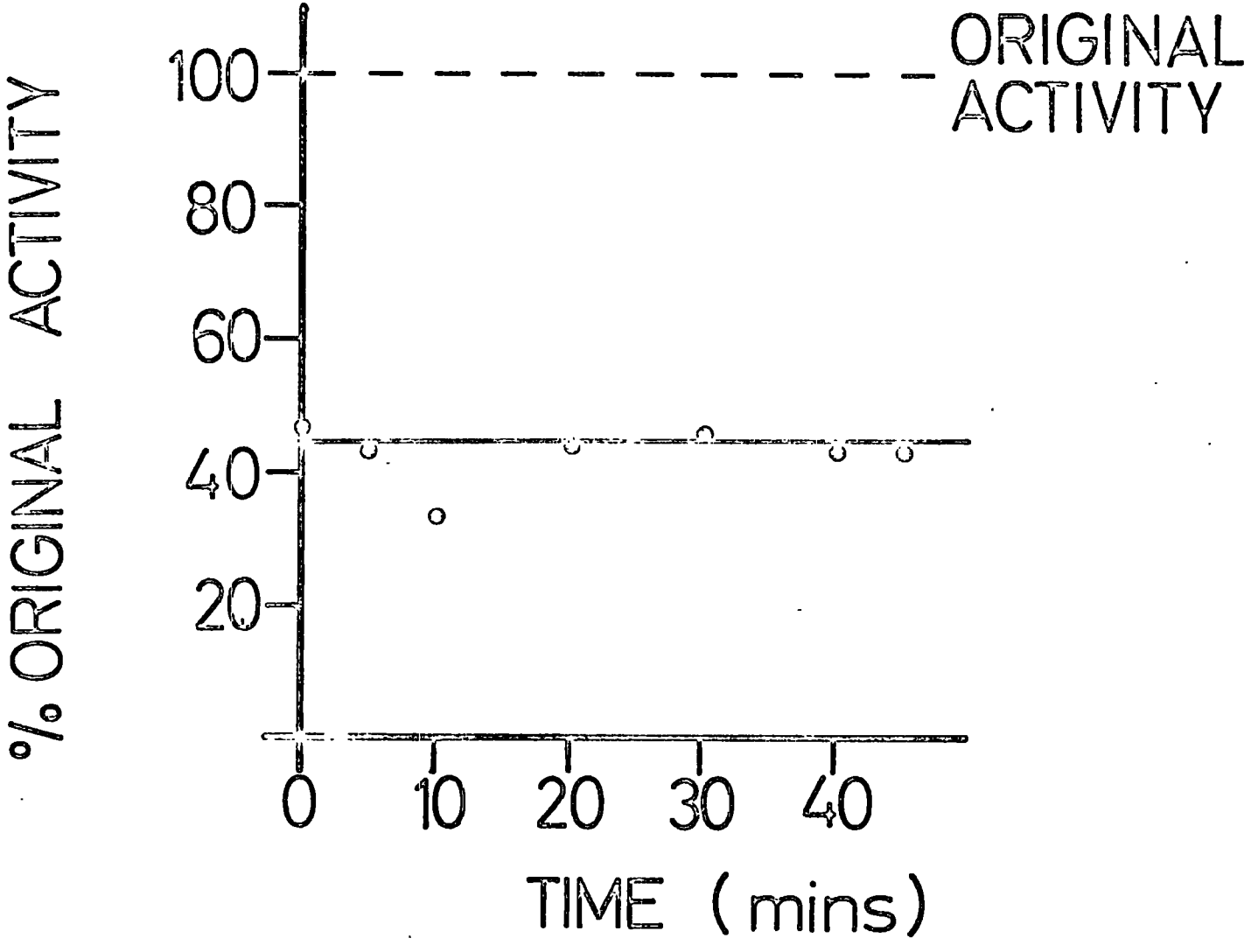

% ORIGINAL ACTIVITY
100
80
60
40
20
ORIGINAL ACTIVITY
0
10
20
30
40
TIME (mins)

Figure 8-8: Effect of dilution upon the thermal inactivation characteristics of Ca^{2+}-ATPase activity

Methods: Each preparation was diluted two-fold and four-fold. The thermal inactivation characteristics of each diluant was determined at 32°C (a) and 33°C (b) in the normal manner.

Legend: Ordinate: % Residual Activity

Abscissa: Preincubation Time at 32°C (minutes)

■ N Concentration

● $\frac{N}{2}$ Concentration

▲ $\frac{N}{4}$ Concentration

Open symbols refer to the preparation treated at 32°C and closed symbols to the preparation treated at 33°C

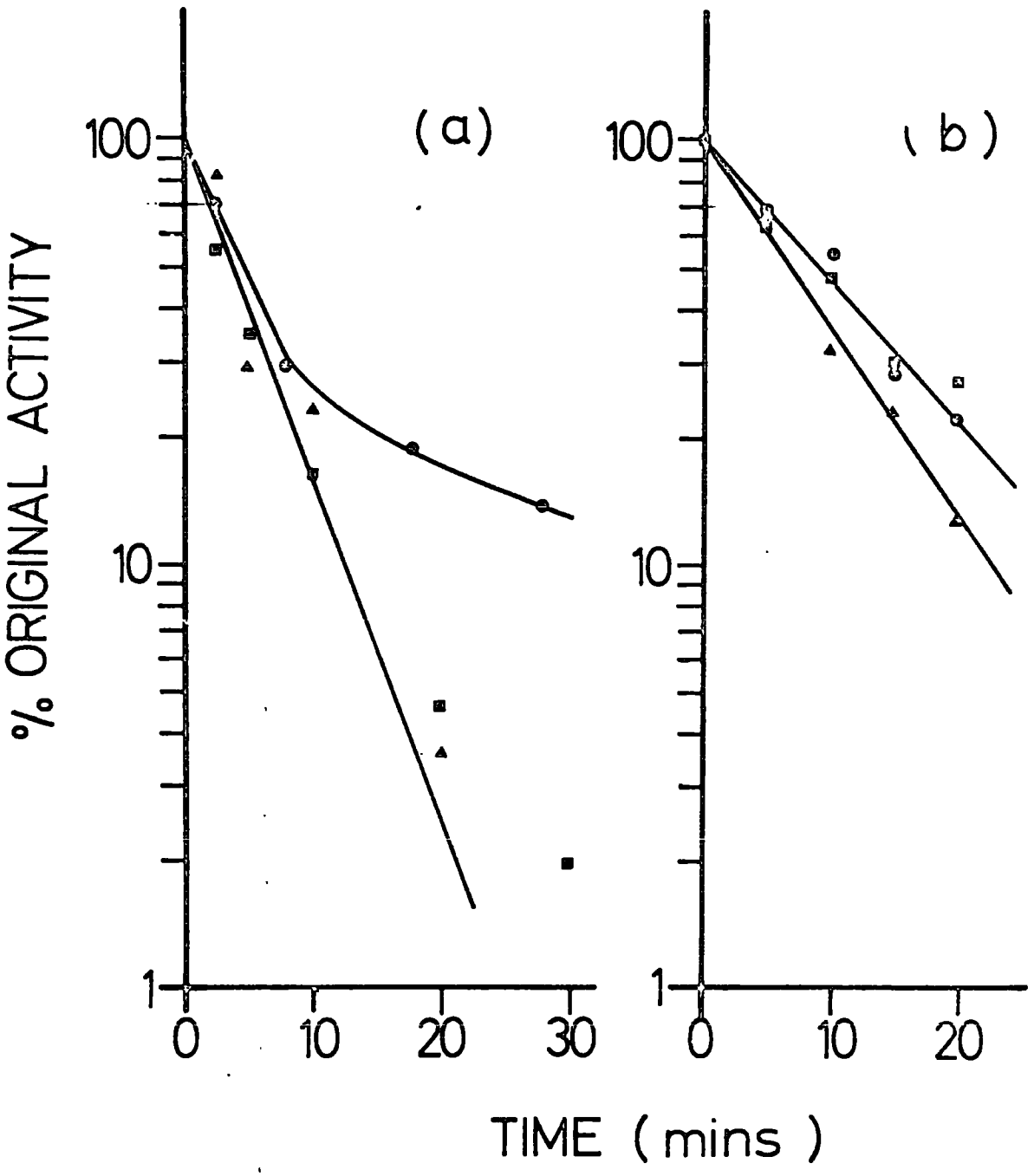

(a)
(b)
100
10
1
% ORIGINAL ACTIVITY
0
10
20
30
TIME (mins)

Figure 8-9: Effect of different concentrations of bovine serum albumin upon the thermal inactivation of the Ca^{2+}-stimulated ATPase

Methods: A microsomal preparation was resuspended in media containing 100, 50, 10 or 0 mg BSA/ml in 5 mM KCl, 10 mM Imidazole-HCl pH 7.1. The thermal inactivation characteristics of each suspension was tested at 32°C in the normal manner.

Legend: Ordinate: % Residual Activity
Abscissa: Preincubation Time at 32°C (minutes)

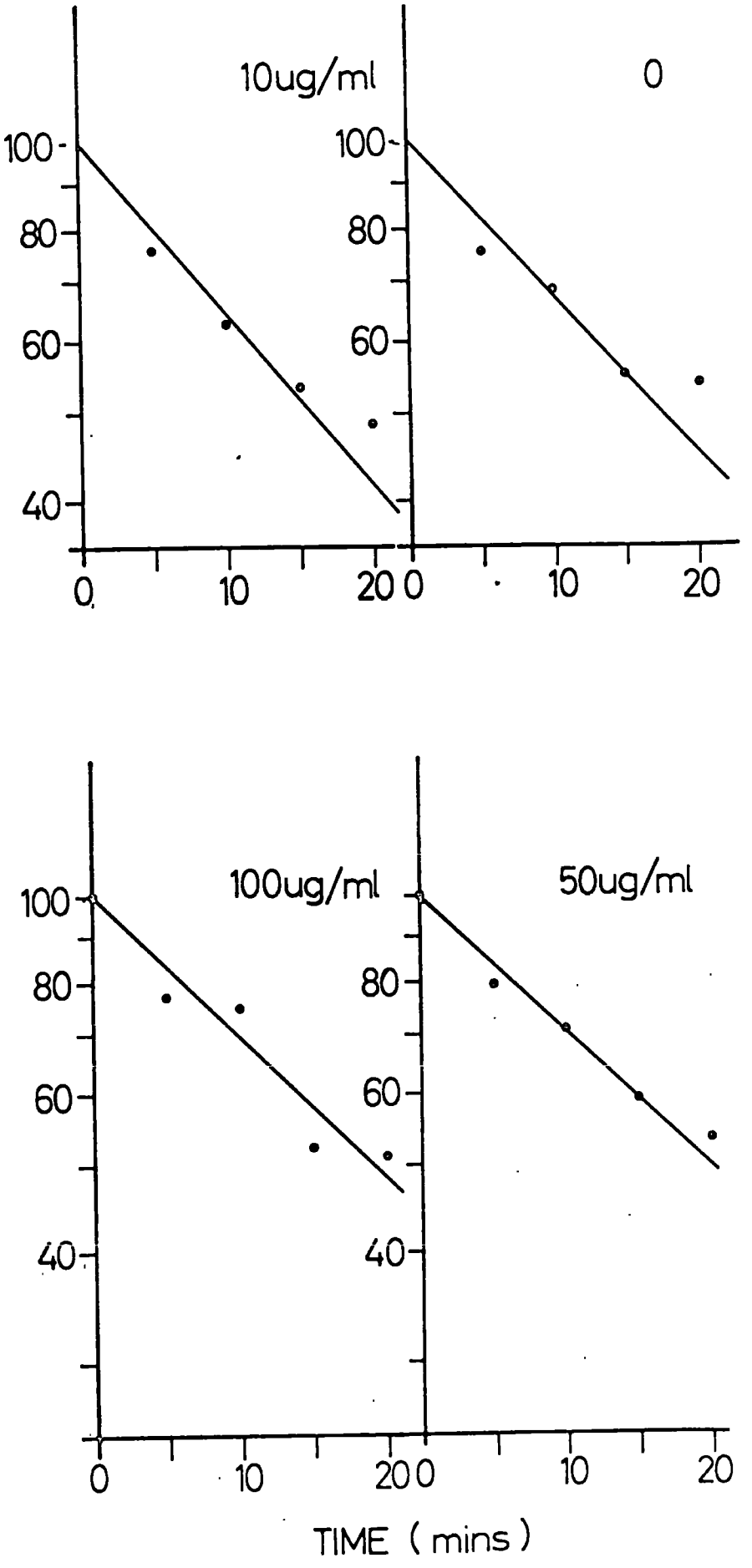

10ug/ml
0
100
80
60
40
0
10
20
100ug/ml
50ug/ml
TIME (mins)

Figure 8-10: Typical inactivation curves for a microsomal Ca^{2+}-ATPase preparation at 31.5-34°C

Methods: A microsomal preparation resuspended in 5 mM KCl, 10 mM Imidazole-HCl, pH 7.1, was treated at 31.5, 32, 32.5, 33 and 34°C for varying periods of time. Residual activity was assayed at 25°C. Values were plotted semi-log form (a) and the log LD_{50} for each preincubation temperature was plotted against temperature.

Legend: Ordinate: % Residual Activity

Abscissa: Preincubation Time (minutes)

□ 31.5°C Preincubation Temperature

▲ 32.0°C

△ 32.5°C

○ 33.0°C

◙ 34.0°C

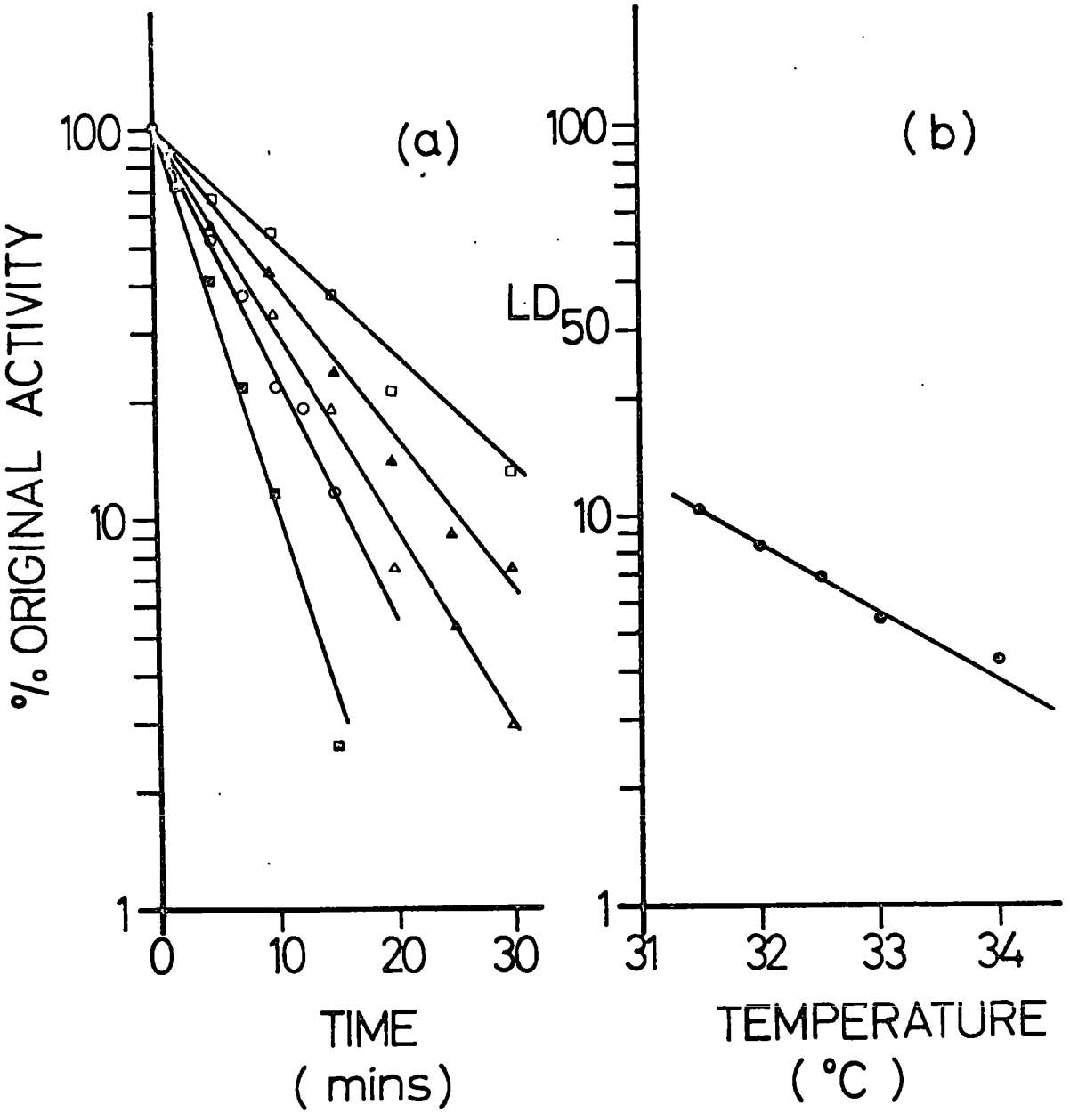

(a)
100
10
1
% ORIGINAL ACTIVITY
0
10
20
30
TIME
(mins)
(b)
100
10
1
LD50
31
32
33
34
TEMPERATURE
(°C)

Figure 8-11: Averaged LD_{50}/Temperature plots of rate of thermal inactivation at each preincubation temperature, for microsomal Ca^{2+}-ATPase preparations isolated from 4°C and 25°C acclimated crayfish

Methods: LD_{50}/Temperature plots for each microsomal preparation were prepared as illustrated in Figure 8-10 (b), and the values for LD_{50} at 31, 32, 33 and 34°C were determined from the best fit line. These values were averaged for preparations from 4°C acclimated crayfish and from 25°C acclimated crayfish (see Table 8-1) and plotted as the mean ± SEM.

Legend: Ordinate: LD_{50} (minutes)
Abscissa: Preincubation Temperature (0°C)
Values plotted are mean ± SEM

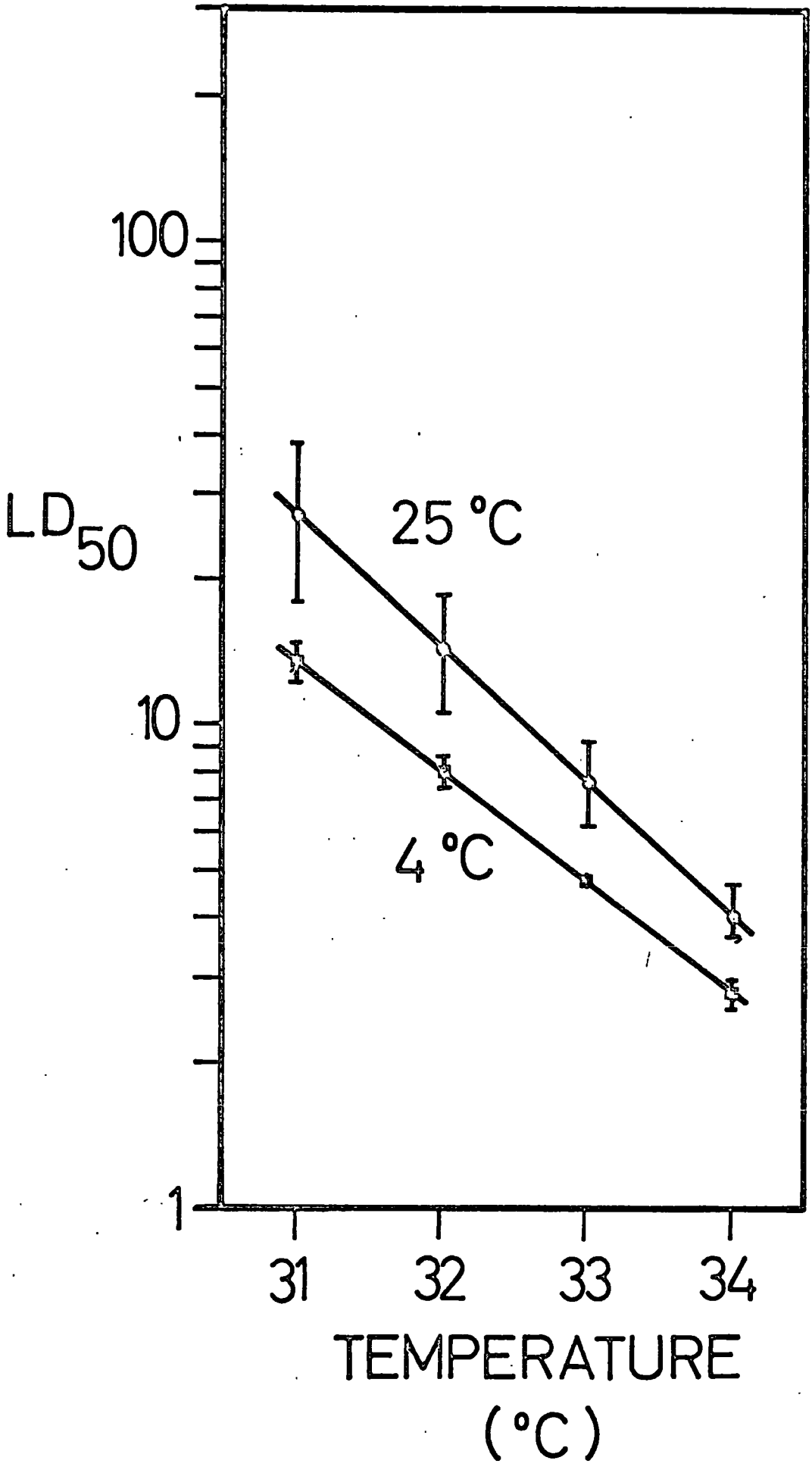

100
10
1
LD50
25 °C
4 °C
31
32
33
34
TEMPERATURE
(°C)

Figure 8-12: Effect of preincubation at 31-33.5°C upon the Mg^{2+}-dependent ATPase activity of a heavy microsomal preparation from crayfish muscle

Methods: A microsomal preparation was preincubated at 31, 32, 32.5 and 33.5°C for varying periods of time. The residual Mg^{2+}-dependent ATPase activity was determined at 25°C and calculated as a % of the activity of an untreated preparation (% Residual Activity).

Legend: Ordinate: % Residual Activity

Abscissa: Preincubation Time (minutes)

31.0°C Preincubation Temperature

32.0°C

32.5°C

33.5°C

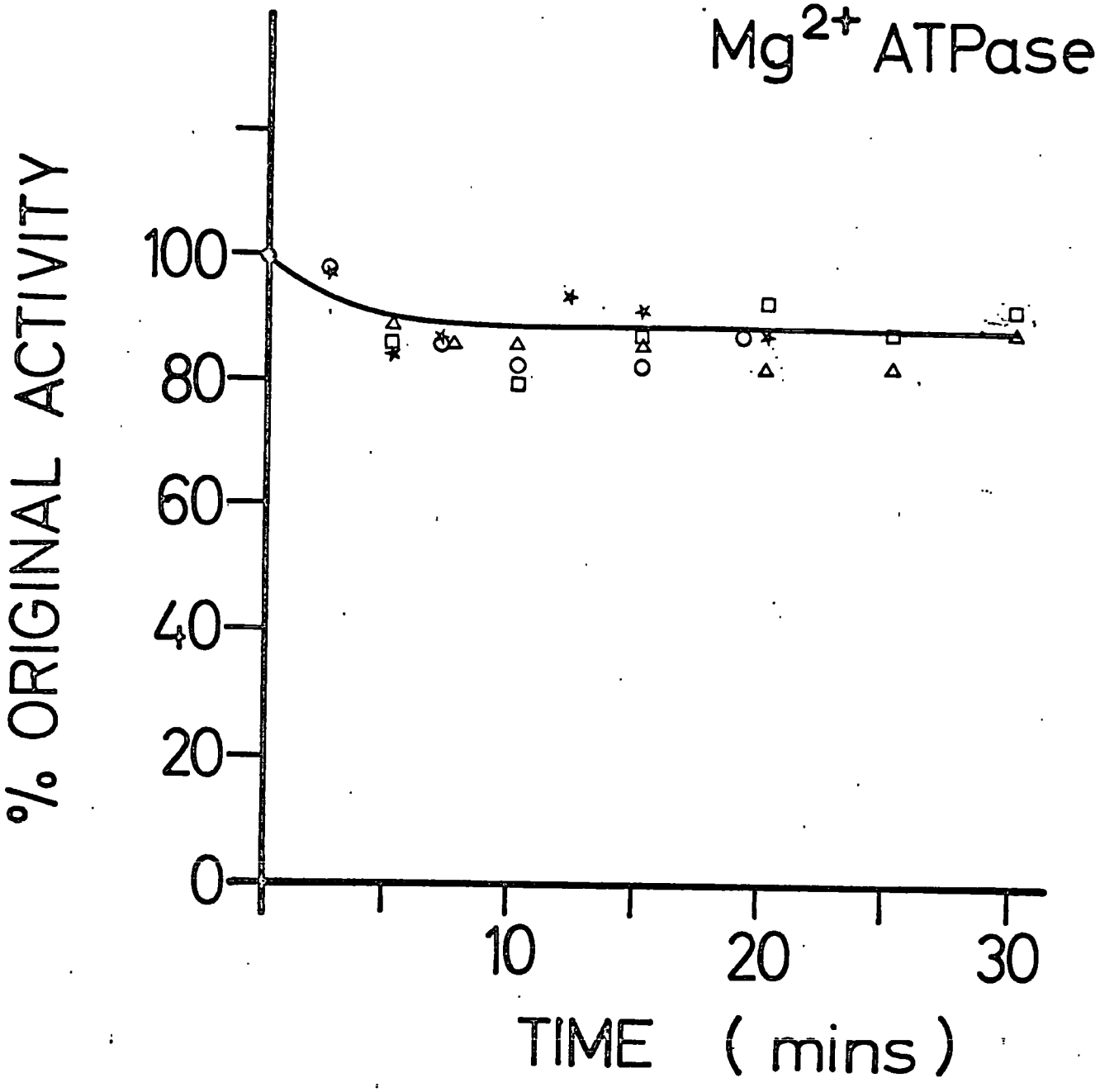
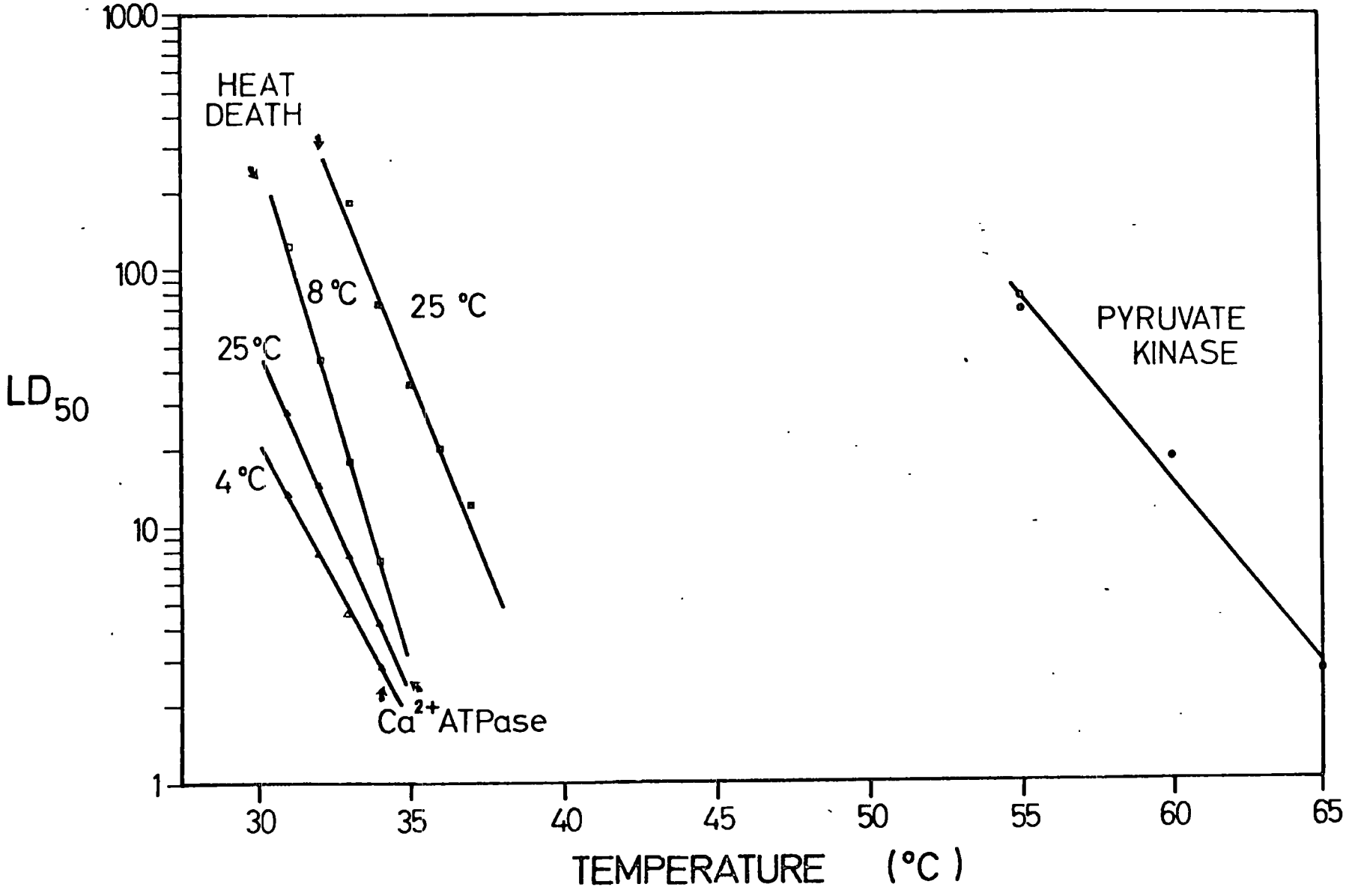

Mg2+ ATPase
% ORIGINAL ACTIVITY
100
80
60
40
20
0
10
20
30
TIME (mins)

Figure 8-13: Comparison of the temperatures of inactivation of the Ca^{2+}-stimulated ATPase of the CSR, and pyruvate kinase, with the process of heat death in the freshwater crayfish

Data for the Ca^{2+}-stimulated ATPase are from Table 8-1. Data for pyruvate are unpublished observations of Cossins. Data for heat death are from Bowler, Duncan and Gladwell (1973).


1000
100
10
1
LD50
HEAT
DEATH
8 °C
25 °C
25 °C
4 °C
Ca2+ATPase
PYRUVATE
KINASE
30
35
40
45
50
55
60
65
TEMPERATURE (°C)

Chapter 9

THE ROLE OF MEMBRANES IN PHYSIOLOGICAL COMPENSATION

GENERAL DISCUSSION

Heat death in Arianta (Grainger, 1969) and in Austropotamobius (Bowler, 1963a; Gladwell, 1973) involves the exchange of large amounts of Na^+ and K^+ between the intracellular and extracellular (haemolymph) compartments of the animal. Bowler and Duncan (1967) suggested that since these ionic movements in Austropotamobius were large, a bulk tissue such as muscle was involved. Indeed, Gladwell (1973) has estimated that skeletal muscle may comprise approximately 50% of the wet weight of a crayfish, and has provided evidence that K^+ is lost from and Na^+ is gained by the muscle during exposure to lethal temperatures. Further electrophysiological studies on the sarcolemma of crayfish skeletal muscle have shown that exposure to high temperature in vitro, caused a progressive increase in the conductance (and permeability) of the sarcolemma, followed by depolarisation of the muscle fibres. In addition, muscle fibres from warm-acclimated crayfish were more resistant to high temperatures than muscle fibres from cold-acclimated crayfish.

Bowler, Duncan, Gladwell and Davison (1973) have suggested that lethal high temperatures in the freshwater crayfish cause a breakdown in the passive permeability barriers of the muscle membrane, and that this is the initial factor in the sequence of events that lead to heat death. (This sequence of events is discussed in detail by Gladwell (1973), and further evidence for the important role of the breakdown in permeability barriers of membranes in cellular heat injury is discussed in Chapter 1.)

The work presented in the present thesis has been concerned mainly with the detailed examination of the biochemical composition and functional properties of crayfish muscle membranes. The aims of this study have been firstly to identify the factor(s) responsible at the subcellular and molecular level for the important breakdown in the permeability barriers of the sarcolemma, and secondly to discover how these factor(s) are modified by thermal acclimation to produce the phenomenon of resistance compensation first observed in Austropotamobius pallipes by Bowler (1963a), and later by Gladwell (1973).

Any potential hypothesis for cellular heat injury must account for several characteristic features of the heat death process (Bowler, Duncan, Gladwell and Davison, 1973). Firstly, the event occurs rapidly over a limited temperature range (30-35°C), and hence has a high temperature coefficient. Secondly, the kinetics of heat death and the temperature range over which it occurs are shifted by thermal acclimation in a compensatory manner.

In the following discussion, an attempt will be made to analyse the various mechanisms that may contribute to the observed breakdown in membrane permeability barriers and to discuss the evidence for and against them. It is clear that this must be done cautiously in view of the many uncertainties associated with our knowledge of cellular and subcellular ultrastructure, and the effects of lethal temperatures upon them. Suggestions for future enquiry will also be made at relevant points.

According to most recent theories, the major constituents of cellular membranes are lipids and proteins, although the arrangement of these components is currently the subject of much discussion (Hendler, 1971; Singer and Nicolson, 1972; Singer, 1974). Davson and Danielli (1943) initially suggested that

cellular membranes are composed of a lipid bilayer with protein adsorbed onto both surfaces and that this structure is the major barrier to the free diffusion of solutes. Indeed, most recent concepts of membrane ultrastructure (Hendler, 1971; Singer and Nicolson, 1972) also ascribe a major structural role to the phospholipid bilayer, although proteins, both enzymatic and otherwise, are incalated within this bilayer to an important extent.

Supporting evidence for the important role of phospholipids in the functional characteristics of natural membranes has been obtained with the use of protein-free, model membrane systems. For example, liposome systems have been shown to mimic successfully the in vivo permeability properties of certain micro-organisms (see Van Deenen, 1972; also Introduction to Chapters 2 and 3). Also indicative of the vital functional importance of phospholipids is the effect of lipid composition upon the regulation of membrane-bound enzyme activity, the lateral motion of membrane constituents, and osmotic stability of cells. (See Chapter 3.)

It has been suggested by Trauble (1971) that uncharged molecules pass through the lipid bilayer by first dissolving in the lipid protein and then diffusing across by following the fluctuations (kinks) in molecular packing. Papahadjopoulos, Nir and Ohki (1972) later suggested that a similar mechanism may account for the permeation of charged species, although the displacement from a medium of high dieletric constant to one of low dielectric constant is thermodynamically unfavourable for charged species in general.

Such diffusion would, therefore, depend upon the occurrence of statistically infrequent 'pores'. Such pores may be the result of fluctuations in the close, molecular packing arrangements of the hydrocarbon chains of the polar lipids which may be caused

by their inherent molecular mobility, the fast, lateral movement of phospholipid molecules along the surface of the membrane, and a slow 'flip-flop' reorientation between the two sides of the bilayer (Hubbell and McConnell, 1971; Kornberg and McConnell, 1971). These effects would become more intense with increased temperature, due not only to the increased molecular mobility of the hydrocarbon chains, but also by a reduction in the London-Van der Waals interactions between them. The net result is a loss of the co-operative restraining influences that promote membrane integrity, and an increase in permeation across the membrane.

Whilst this process as outlined is not precisely the same as that proposed in the 'lipid liberation theory', it has the same end result, namely, a breakdown in the structural integrity and functional properties of a cell, and an interruption of the normal physiological and metabolic processes of the cell.

Van Deenen and his colleagues have repeatedly shown that the permeability of non-electrolytes and electrolytes from 'liposome' model membrane systems (Demel, Kinsky, Kinsky, Van Deenen, 1968; de Gier, Mandersloot and Van Deenen, 1968; Demel, Geurts van Kessel and Van Deenen, 1972; Papahadjopoulos, Nir and Ohki, 1972), from cells of Acholeplasma laidlawii (De Kruyff, Demel and Van Deenen, 1972; McElhaney, de Gier and Van der Neut-Kok, 1973), E. coli (Haest, de Gier and Van Deenen, 1969) and Staphylococcus aureus (Haest, Op den Kamp, Bartols and Van Deenen, 1972) is markedly dependent upon temperature with an activation energy of between 18-30 Kcals $mole^{-1}$. According to McElhaney, de Gier and Van der Neut-Kok (1973) the value of activation energy for the permeability of non-electrolytes is unaffected by the hydrocarbon chain composition of the phospholipids or the cholesterol content of Acholeplasma cells and their derived liposomes, and depends only upon the structure of the permeating molecule. The activation energy for the permeation of monovalent cations above the transition

temperature is 13-17 Kcals $mole^{-1}$ from liposomes (Papahadjopoulos and Watkins, 1967) and erythrocytes (Glynn, 1956). The lower Ea characteristic of the permeation process at normal temperatures, compared to the high Ea characteristic of heat death (see Chapter 8 may appear to be an argument against its role in the latter process. However, it should be remembered that heat death is almost certainly a complex process and the overall Ea is probably a reflection not only of the primary lesion of heat death but also of the secondary and teriary events that occur during the subsequent breakdown of homoeostasis. There have been few studies on the permeation process at temperatures that cause heat death. Papahadjopoulos, Nir and Ohki (1972) discuss the possibility that small, reversible, temperature-dependent changes in the lipid bilayer could account for an increase in the slope of Arrhenius plots at higher temperatures. Singer and Nicolson (1972) have described certain co-operative phenomena as an effect which is initiated at one site on a complex structure and transmitted to some remote site by some structural coupling between the two sites. They describe several such effects which have large scale effects which involve the long-range transmission and amplification of localised events over the entire surface of a membrane. Thermal disruption of membrane integrity at heat death temperatures may be another rather extreme example of this phenomenon which, due to its co-operative nature, may have a ΔH^* which approaches that characteristic of heat death.

The involvement of membrane lipids in resistance compensation is supported by the observations in Chapter 3, which provide clear evidence for changes in the biochemical composition of muscle phospholipids in response to changes in environmental temperature. This provides a mechanism for the effects of temperature upon permeability and stability of the sarcolemma (Gladwell, 1973) of the freshwater crayfish.

It has been suggested previously that poikilothermic organisms and micro-organisms possess a feedback mechanism linked to the environmental temperature which enables the fatty acyl composition of membrane phospholipids to be altered in order to maintain a constant and presumably optimal membrane fluidity. The mechanism of control of this servomechanism remains obscure, although the effect of photoperiod upon lipd composition suggests an important role for humoral factors (see Chapter 3).

Until recently the mechanism for changing the acyl composition of membrane lipids remained obscure. A number of possibilities exist which are most easily understood by reference to Figure 9-1, which outlines the important steps in the biosynthesis of membrane phosphoglycerides. Specificity with regard to fatty acid incorporation may be exercised at a number of points in this sequence, namely, at the acylation, phosphorylation and base-incorporation steps, or by controlling the enzyme systems responsible for these reactions in response to temperature. Lands and his colleagues (Lands, 1964) have provided evidence that 1-acyl transferase enzymes possess a considerable degree of specificity with regard to their acyl substrate. Sinensky (1971) has shown that the proportion of oleate to stearate incorporated from the medium into E. coli phospholipids increased with decreasing temperature. Temperature was shown to affect the relative activity of glycero-3-phosphate and monoacyl-glycerol-3-phosphate acyltransferases, rather than the net rate of synthesis or degradation of the enzyme.

These mechanisms, however, do not apply to all organisms and a direct effect of temperature upon unsaturated fatty acid synthesi seems to be a more common means of changing membrane composition, particularly in microorganisms. Fulco (1974) has intensively investigated the temperature-mediated control of desaturation in Bacillus megaterium, and has demonstrated three control mechanisms which regulate the level of desaturating enzymes and the rate of

unsaturated fatty acid biosynthesis in response to temperature changes. These are (a) desaturase induction at low temperatures, (b) irreversible, first-order inactivation of a desaturase at high temperatures, and (c) zero-order decay of desaturase synthesising system at high temperatures.

In higher animals the effects of diet have been investigated, as well as the roles of age, levels of acyl receptors and circadian rhythms, all of which may complicate the response to temperature changes (for review see Fulco, 1974). Harris and James (1969a, b) have shown that in certain plant tissues at least, the effect of temperature can best be explained in terms of an increased oxygen solubility as the temperature decreases, since oxygen is a substrate for the desaturase reaction and may be rate-limiting.

It is significant that Gladwell (1973) reports a fall in the muscle membrane resistance on treatment at 32°C. He suggests that this is due to a dramatic increase in Na^+ permeability that also occurs. The associated redistribution of K^+ across the membrane would also contribute to the fall in resting potential. The demonstration that the resting potential returns to its normal value when heat-treated muscle is bathed in cold saline of 'normal' ionic conditions, indicates that the increase in permeability caused by the high temperature is reversible. It also suggests either that the intracellular K^+ concentration of the heat-treated muscle preparation had not fallen greatly, or perhaps that the 'Na^+ pump' was still efficiently restoring the normal intracellular values of Na^+ and K^+. In either case, such a phenomenon is unlikely to be explained in terms of an irreversible process such as protein denaturation.

As pointed out in Chapter 4, a major advantage of the FSR preparation is that both the transport and ATPase functions may be studied. Deamer (1973) has noticed an intriguing effect of temperature upon the Ca^{2+} uptake mechanism of the FSR preparation

of lobster abdominal muscle. As the temperature rises both ATPase and Ca^{2+} uptake by the vesicles increase in accordance with the Van't-Hoff, Arrhenius laws, the Ca^{2+}/ATP ratio remaining constant. However, at temperatures above 30°C, the Ca^{2+}-uptake function rapidly deteriorates although the ATPase activity continues to exhibit increased activity. This apparent "uncoupling" of ATPase activity from calcium sequestration is probably explained by the inability of the vesicle to retain accumulated Ca^{2+}, due to a vastly increased membrane permeability. Indeed, this behaviour has been confirmed in rabbit FSR by Eletr and Inesi (1972) and Johnson and Inesi (1969) although, as one might expect, it occurs at a higher temperature in this preparation. It would be of great interest to see if a similar situation exists in crayfish FSR, since this would constitute direct evidence for an increase in muscle membrane permeability at heat death temperatures. In addition, the effect of thermal acclimation on this phenomenon could be studied although Tume, Newbold and Horgan (1973) maintain that changes in the fatty acid composition of rat skeletal muscle FSR caused by feeding fat supplemented or deficient diets had no effect upon such properties of the preparation as rate of Ca^{2+} uptake, total amount of accumulated Ca^{2+}, the rate and extent of Ca^{2+} release in the cold, and the 'basal' and 'extra' ATPase activities.

The denaturation of intrinsic, membrane-bound proteins is a second potential mechanism for the disruption of membrane integrity and is seen as a property of both lipid and protein. The unfoldin of the three-dimensional form of protein molecules that is thought to occur during this process, may have large-scale and long-range effects upon the liquid-crystalline arrangement of the surrounding lipid matrix, presumably by physical interruption of their close, stearic packing arrangements which, as explained previously, will result in a reduction in the stability of the membrane.

Alternatively, the protein moeity of the membrane may create 'pores' in the membrane which, presumably, would be lined by hydrophilic amino-acid residues. Such aqueous pores will clearly lead to a vast reduction in the energy requirement for ion movement across the membrane, since it becomes a process of diffusion through aqueous channels, although it may be hindered or encouraged by the presence of fixed charges.

Such changes in protein configuration are irreversible, and as explained earlier cannot explain the reversible nature of the permeability increase. Other evidence concerning the Ca^{2+} stimulated ATPase of crayfish FSR (Chapter 8), and the (Na^+,K^+)-ATPase, and Mg^{2+} ATPase of the sarcolemma (Bowler and Duncan, 1967; Gladwell, 1973; Bowler, Duncan, Gladwell and Davison, 1973) indicates that these enzymes are not inactivated <u>in vivo</u> during heat death. However, the marked increase in Km(ATP) for Ca^{2+}-stimulated ATPase activity at 35°C (see Chapter 6), does indicate a deleterious effect of this high temperature upon enzyme-substrate affinity. Presumably, temperature causes some conformational modification of the enzyme molecule that impedes enzyme-substrate interactions. Enzyme activity itself is not affected by exposure to 35°C, since ATP hydrolysis was linear with time at this temperature.

The denaturation characteristics of a membrane-bound protein may be an intrinsic property of the protein molecule itself, or may be dependent upon the viscosity of its solvent environment. This latter alternative is favoured by the observation of irreversible inactivation of several membrane-bound ATPases from crayfish muscle over the limited temperature range of 30-35°C. Soluble enzymes by contrast are usually inactivated at temperatures well above those characteristic of heat death (e.g., pyruvate kinase from crayfish muscle, Figure 8-13; see also Ushakov, 1964, 1966, 1967; Read, 1967). Again, it is emphasised that these

phenomena only occur under rather special ionic conditions, and for reasons enunciated in Chapter 8 probably do not occur during heat death *in vivo*.

A major obstacle to the hypothesis that *in vitro* thermal inactivation characteristics of a membrane-bound enzyme is a reflection of the kinetic state of its hydrophobic environment, is the lack of any acclimation effect upon the rate of inactivation of the Ca^{2+}-ATPase of crayfish FSR. Experimental observations of Tume, Newbold and Horgan (1974) also suggest that the calcium sequestration machinery in rabbit FSR preparations is insensitive to the gross fatty acid composition of its lipid environment. Similarly, the results presented in Chapters 6 and 7 show that the Ca^{2+}-stimulated ATPase of the FSR was not affected by the acclimation history of the animal since the response of enzyme activity to temperature was identical in preparations isolated from $4^{o}C$ and $25^{o}C$ acclimated animals. In addition, the reaction characteristics of the Ca^{2+}-stimulated ATPase from both acclimation groups were not significantly different. These studies suggest that this particular enzyme is not influenced by fatty acid composition of membrane lipids, and as such does not reflect the state of fluidity of the membrane lipids as many others are known to do (Coleman, 1973). However, it should be remembered that the lipid analysis described in Chapters 2 and 3, was performed on the total lipid fraction from crayfish muscle, and gives no definite information on the effect of acclimation upon FSR membranes. Clearly, an examination of the effects of thermal acclimation upon the lipid composition of crayfish FSR would clarify the situation.

Gladwell (1973) and Bowler, Duncan, Gladwell and Davison (1973) maintain that the Mg^{2+}-ATPase from the muscle of cold-acclimated crayfish is inactivated at a faster rate than the corresponding enzyme from warm-acclimated crayfish. However, the precise nature of the Mg^{2+}-dependent ATPase activity studied

by these workers is not clear, since it is evident that a number of membrane and non-membrane contaminants were present. Chief amongst these is the Mg^{2+}-ATPase activity associated with myofibril since no special procedures were employed in these studies to exclude this source of contamination. Indeed, there are several other studies that indicate that the myofibrillar ATPase from various species are inactivated at temperatures only just above the tolerated range. Licht (1967) has demonstrated a close relationship between the enzymatic thermal optima of myofibrillar ATPase activity and the preferred body temperature for a number of reptiles. In addition, heavy microsomal preparations isolated from crayfish muscle that are heavily contaminated with myofibrilla material shows a more rapid rate of inactivation at heat death temperatures than the pure FSR preparation, whereas the Mg^{2+}-ATPase of the FSR preparation was completely stable at heat death temperatures (Cossins and Bowler, unpublished observation). It is therefore suggested that the enzyme studied by Bowler and his colleagues was not entirely of sarcolemmal origin and probably does not represent the mechanism of control of membrane permeabilit properties. However, Ushakov (1967) has been unable to demonstrate changes in the thermostability of saurian myofibrillar ATPases after thermal acclimation in the same way that the Mg^{2+}-ATPase of the crayfish studied by Bowler and his co-workers shows increased thermal resistance in warm-acclimated individuals compared to cold-acclimated animals.

In conclusion, it may be stated that the most attractive hypothesis accounting for cellular heat injury in Austropotamobius pallipes, is that concerning the membrane lipids. It has been proposed earlier that the breakdown of membrane permeability properties during heat death is the sole property of the polar lipi portion of the sarcolemma. Increased molecular mobility of the hydrocarbon chains of these polar lipids may lead to a co-operative

breakdown in the integrity of the lipid bilayer. The dynamics of breakdown in membrane integrity is envisaged as being somewhat dependent upon the biochemical composition of the membrane lipids and hence the acclimated state of the animal. This hypothesis can, with certain reservations, explain the criteria outlined previously, namely, occurring over a limited temperature range (high Ea) and an acclimation effect. In addition, this phenomenon is in accord with the observation by Gladwell (1973) that the loss of permeability control is reversible in vitro.

It appears that membrane ATPases at least, are not denatured during the heat death process and on present evidence on their contribution to heat death, must be discounted.

Finally, the proposed hypothesis has interesting implications for the relationship between resistance compensation of the crayfish and capacity compensation of certain physiological processes that are dependent upon the state of the membrane. It has been pointed out previously (Chapter 1) that the relationship between these two compensatory processes is not clear. The notion that heat death in the freshwater crayfish is caused primarily by a breakdown in the physical integrity of the membrane, and that this breakdown is dependent upon the degree of unsaturation of the membrane lipids, suggests that resistance compensation in this instance is a consequence of alterations of the fatty acid constituents of the membrane. Such biochemical modifications in membrane lipids (see Chapter 3) also result in compensatory changes in the functional properties of membranes in the normal viable temperature range, such as permeability, transport of metabolites, effects upon membrane-bound enzyme and the lateral motion of membrane constituents, As such the two processes may be consequences of the same biochemical modification.

Unfortunately, the Ca^{2+}-stimulated ATPase of the crayfish FSR preparation did not show either capacity compensation

(Chapters 6 and 7) or resistance compensation effects (Chapter 8). These results and others (e.g., Tume, Newbold and Horgan, 1974) suggest that the Ca^{2+}-stimulated ATPase is relatively insensitive to the physical state of their hydrophobic environment.

The same situation may apply to modifications in protein structure. Hochachka and his colleagues (Hochachka and Somero, 1971, 1973) have shown at the molecular level how changes in enzyme machinery of the cell may lead to thermal compensation of their activity. It is apparent from various studies on enzyme inactivation (Ushakov, 1964, 1966, 1967; Read, 1967) that 'soluble enzymes are denatured at temperatures well above those characteristic of heat death of the whole animal. Presumably they are not directly involved in the process of resistance compensation. However, it is also apparent that the temperature regime that is normally experienced by an enzyme may be correlated with its inactivation temperature (Ushakov, 1964, 1967). Enzymes are thought to undergo conformational changes during activity and these must occur at the environmental temperature. It follows that an enzyme which functions efficiently in a cold environment will be more flexible, at any one temperature, than the corresponding enzyme which functions at warmer temperatures, and presumably would be more susceptible to elevated temperature. Thus changes in enzyme structure during evolutionary adaptation or seasonal compensation to different thermal regimes, may result in both capacity compensation of enzyme function, and resistance compensation.

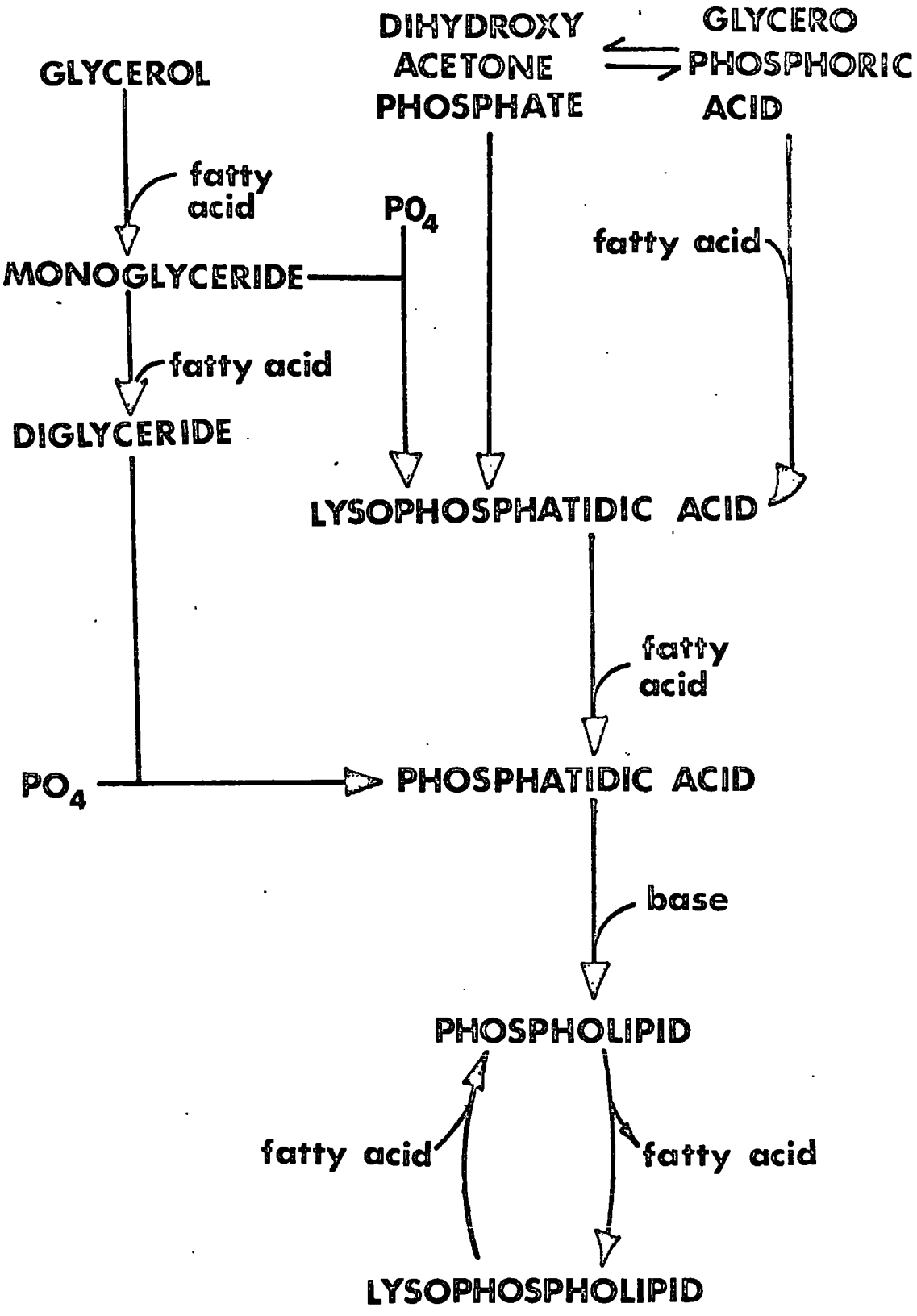


Figure 9-1 Simplified Scheme for the Biosynthesis of Phospholipids

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Appendix:

THELOHANIA CONTEJEANI

Microsporidian Parasite of Crayfish Muscle.

INTRODUCTION

The drastic effects of the Crayfish plague fungus Aphanomyces astaci on crayfish populations throughout Northern Europe ensures that crayfish diseases are treated with the utmost respect. Hence the occurrence of Thelohania contejeani in our population was studied to determine its effect on local crayfish populations and on the infected individual. Crayfish parasitized by T. contejeani are characterized by the opaque, white appearance of the abdominal muscle, as compared with the more translucent appearance of normal muscle when seen from the ventral surface. Internal examination showed that all skeletal muscle had this opaque, white appearance. In an advanced infection the animal was noticeably sluggish and the tail-flick escape reaction was somewhat ineffectual. Both juvenile and adult animals of both sexes were found infected.

T. contejeani has been reported to infect two European crayfish, Astacus astacus (Schäperclaus, 1954; Voronin, 1971; Sumari and Westman, 1969) and Austropotamobius pallipes (Dollfus, 1935; Pixell Goodrich, 1956; Vey and Vago, 1973). It is known to be prevalent throughout Northern Europe with recent observations recorded in Russia (Voronin, 1971), Finland (Sumari and Westman, 1969), Germany (Schäperclaus, (1954) and France (Vey and Vago, 1973). The disease has

been recorded earlier by Pixell Goodrich (1956) in a population of crayfish from Oxfordshire and parasitized individuals have been observed in crayfish supplied from the Haslemere region of Surrey and from the River Derwent, Derbyshire, as well as in stocks caughts in Northumberland, England, by ourselves. It is probable that this disease is a more or less permanent feature in crayfish populations of England.

The observations reported here concern the histological and ultrastructural characteristics of the parasite.

MATERIALS AND METHODS

Austropotamobius pallipes Lereboullet were caught in autumn 1971, and summer 1972 in the Whittle Dene, Hallington and Matfen reservoir complexes in Northumberland, U.K. Crayfish were maintained in the laboratory in well-aerated water at 15°C and fed with fragments of fish, potatoes and tropical fish food.

Tissues of infected animals were fixed in Bouin's solution, embedded in paraffin wax, sectioned (8 μm) and were stained with either Heidenhain's iron haematoxylin or Halmi's mixture (Ewen, 1962).

Smaller fragments of tissue (3 x 1 x 1)10^{-3} m were fixed at room temperature in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 1 h and post-fixed in 1% osmic acid in the same buffer. The fixed material was embedded in

Epon and sectioned with a Reichert Om U2 Ultratome. Thick sections (1 μm) were taken and stained with a 1% Toluidine blue solution in 1% aqueous borax, for light microscopy. Ultra-thin sections were stained with 1% uranyl acetate in 70% ethanol and poststained with lead citrate (Reynold, 1963) for electron microscopy. Sections were examined using an A.E.I. EM801 electron microscope. Spores were very electron-dense when stained with both lead citrate and uranyl acetate. However, uranyl acetate alone gave adequate resolution of internal spore structures, but with reduced electron density. Although the preparative technique was rigorously adhered to, variation occurred in the quality of fixation of certain protozoan stages, even though adjacent host tissue was well fixed.

Both fresh and fixed smears of living parasites were examined. The latter were fixed in methanol and stained with Giemsa solution.

Histological examination

The work presented here is the result of histological and electron microscopical examination of 18 of the 45 infected individuals, of the 552 animals trapped.

Halmi's mixture was found to be a particularly useful stain because parasitized muscle stained orange, whereas normal muscle was green. Using this and other techniques, it

was possible to show the presence of parasitized fibres, not only in the abdominal flexor muscle (Pl. 1A) but also in the eyestalk, gastric musculature, limb muscles and the longitudinal muscle fibres of the hind gut and cardiac stomach (Cossins, 1973).

The only other tissue found infected was the supra-oesophageal ganglion, where cell bodies occasionally contained small spore masses. Axons in the optic lobe and ventral nerve cord were free from the parasite. Spores were identified extracellularly in the haemocoele of the gill and hepatopancreas.

Phase-contrast examination of fresh muscle smears revealed the presence of several stages in the life-cycle of T. contejeani. The most numerous stage was the mature spore. These were oval, 3.5-4.0 μm long and 1.8-2.0 μm broad (Fig. 1A). This agrees well with the data presented by Pixell Goodrich (1956). A small percentage of spores were aberrant (Fig. 1B). All possessed a clear vacuole at one pole, although it was not possible to relate this to the ultrastructural features of the spore. The nucleus was seen as an elongated or double structure when stained with Giemsa solution (Fig. 1C, D).

The polar filament was everted by mechanical pressure and was most conveniently viewed in an area where the fluid between the slide and coverslip had receded. It varied in

length up to a maximum of 55 μm (Fig. 1L).

Other less refractive bodies found in muscle homogenates were presumed to be vegetative and sporogonic stages of the parasite. They could be broadly grouped into two categories: (1) Spherical, rather opaque structures, about 4-5 μm in diameter, containing one (Fig. 1E) or two (Fig. 1F) nuclei. This is thought to represent the schizont stage. (2) Also spherical but considerably larger in diameter (8-9 μm). With Giesma-stained smears these were observed to have two, four or eight nuclei (Fig. 1G-I) arranged in pairs. They also exhibited a small clear vacuole and one or two dense granules within the cytoplasm. These were thought to be sporoblast stages.

Pansporoblasts were roughly spherical, contained eight spores enclosed within a distinct membrane and were about 8-9 μm in diameter. Immature spores, characterized by the absence of a vacuole were more tightly grouped (Fig. 1J) within the pansporoblast than were the mature spores (Fig. 1K).

Ultrastructural observations

Abdominal flexor muscle preparations of uninfected A. pallipes showed the features reported by Brandt, Reuben, Guardier and Grundfest (1965) in Procambarus sp. Ultrastructural studies of parasitized muscle fibres (Pl. 1B) confirmed the light microscope observations that a large

spore mass occupied the centre of the infected muscle fibre. Normal myofibrils were pushed to the periphery beneath the sarcolemma. These myofibrils, their accompanying membrane systems and neighbouring muscle fibres appeared ultrastructurally normal. No lysis or disintegration was apparent. The central spore mass consists mainly of individual spores (Pl. 1C) separated by an interstitial ground substance composed of small vesicles, electron-dense granules and some disorganized myofibrils undergoing lysis. Occasionally other stages in the life-cycle of T. contejeani were evident within the spore mass. The collagen sheath surrounding the muscle-fibre appears to remain intact. Indeed, it was often the only recognizable host structure.

The spores of T. contejeani were similar to those observed in other Microsporidian species (Huger, 1960; Kudo and Daniels, 1963; Lom and Corliss, 1967; Sprague and Vernick, 1969; Jensen and Wellings, 1972). They were ovoid, electron-dense bodies measuring about 3.3 x 1.5 µm (Pl. 2A), the spores having shrunk during fixation. The spore capsule was 0.09 µm thick (Pl. 2C), except adjacent to the basal portion of the polar filament where it was 0.04 µm thick (Pl. 2E). It was composed of an inner electron-lucent layer and an outer more electron-dense layer. No spore appendages or bristles were observed (Vavra, 1965; Sprague, Vernick and Lloyd, 1968). A spore membrane lies apposed to the inner surface of the

capsule (Pl. 2C). External to the capsule is a single crenelated membrane, probably the remnants of the sporoblast plasmalemma.

The polar sac was seen at the anterior pole beneath the spore membrane (Pl. 2E). Centrally it formed a dome covering the swollen base of the polar filament, to which it was closely attached. It extended peripherally as a flattened membrane-bound sac to enclose the anterior portion of the polaroplast (Jensen and Wellings, 1972). The polar filament passed posteriorly and was thrown into a series (usually 6 or 7) of coils in the posterior third of the spore (Pl. 2A). The coiled polar filament was seen in transverse section (Pl. 2C) as a tubular membrane-bound structure, approximately 0.12 μm in diameter. Internally it contained an electron-dense ring and an electron-dense core. The uncoiled portion was appreciably larger, about 0.18 μm in diameter.

Surrounding the base of the polar filament and occupying the anterior third of the spore was a complex system of membranes (Pl. 2E), the polaroplast. The anterior half was composed of a series of delicate lamellae, closely apposed to each other and lying parallel to the external spore capsule. The posterior half was also composed of membranes but they lay transversely across the spore and were well separated by electron-dense material.

In some spores a double structure was evident in the central area (Pl. 2B). By comparison with similar structures in other stages of the life-cycle of the parasite it is suggested that this represents the nuclear apparatus in its characteristic double form (Vey and Vago, 1973). The margins of these structures were indistinct, although they do suggest the presence of a membrane. The remainder of the central part of the spore was filled with an electron-dense granular sporoplasm.

At the posterior pole of the spore and lying internal to the coiled polar filament was a large vacuole, the posterior vacuole. It was occupied by numerous loosely packed granules and a granular membranous sac. During fixation this vacuole usually subsided leading to collapse of the spore capsule external to it (Pl. 2B).

Most pre-pansporoblast stages of the parasite conformed to the same basic plan as shown in Pl. 2F. Such 'diplocarya' (Vavra, 1965) occurred frequently throughout the spore mass and were often grouped in clusters. They were usually spherical or elongated in section and varied in size from 4 to 7 μm diameter for the spherical forms and 2.5-4 μm x 6-7 μm for the elongate forms. All possessed a distinct plasmalemma. There was no evidence of schizonts possessing intimate host-parasite cytoplasmic relationships as observed in Glugea sp. by Sprague and Vernick (1968).

Each diplocaryon appeared to be binucleate, although serial sections would be needed to confirm this. The nuclear apparatus was characterized by the presence of a double nuclear membrane separated by a clear peri-nuclear space. Centrally these membranes were closely apposed to form a very electron-dense line (Pl. 2F). Other schizonts appeared uninucleate in section.

Microtubules of the spindle apparatus were apparent in the nucleoplasm of several schizonts (Pls. 2F, 3A). In the latter plate two groups of spindle fibres may be seen, lying roughly parallel to the electron-dense nuclear membranes such that the nuclei are dividing to form two nuclear pairs.

Evidently, the nuclear membranes remain intact during nuclear division. The microtubules converge on to an electron-dense region of the nuclear membrane, which is clearly indented and has a small, closed, membranous sac external to it. This complex closely resembles that found in T. bracteata (Vavra, 1965), Nosema nelsoni (Sprague and Vernick, 1969) and Glugea sp. (Sprague and Vernick, 1968). Vavra (1965) has suggested that it carries out the function of the centriole but lacks its characteristic structure.

Whereas the nuclear structure was relatively uniform in all schizonts, considerable variations existed in cytoplasmic detail. At least three forms became apparent. In the first form the schizont possessed many cytoplasmic granules, which

approximate in size to eucaryote ribosomes, and a smooth but underdeveloped endoplasmic reticulum (Pl. 3B). Other schizonts were typified by a smooth, endoplasmic reticulum organized into numerous, parallel, flattened sacs (Pl. 2F). The third form exhibited a mass of small, membranous sacs within the cytoplasm (Pl. 3C). Whilst it is not possible to arrange these forms into any sequence it is tempting to suggest that they do form a developmental progression. No other cytoplasmic organelle, mitochondria nor typical Golgi apparatus has been seen in these stages.

Plate 3D shows two binuclear structures arranged side by side, but there is no cytoplasmic division. It is suggested that this stage represents an early sporont stage, for in other sections parasites with four binuclear structures within a distinct plasmalemma have been observed.

The pansporoblast characteristically contains eight spores, but under the electron-microscope up to five mature spores only could be seen in any one section (Pl. 3E). The pansporoblast was spherical, about 8-9μm diameter and was surrounded externally by a distinct plasmalemma. Internally the only contents other than spores were small electron-dense granules and a mass of unidentified tubular structures (of variable diameter and up to 1 μm in length) (Pl. 2D). The latter are similar to those described in many other Microsporidian species (T. bracteata in Vavra (1965), Glugea sp. in Sprague,

Vernick and Lloyd (1968)). Whereas the electron-dense granules may be seen surrounding the spores after the pansporoblast membrane has disintegrated (Pl. 1C), the tubules have never been observed exterior to a recognizable pansporoblast.

In several preparations late sporoblast stages were observed which were not enclosed within a pansporoblast membrane (Pl. 4A, B). The stages were characterized by significant shrinkage away from the surrounding cytoplasm during fixation, forming an electron-dense body with a crenelated margin. These structures are similar to those observed in Glugea sp. by Sprague and Vernick (1968), except that a binuclear structure was clearly seen.

In another preparation it was possible to observe a sporont undergoing cleavage to form, presumably, eight sporoblasts (Pl. 4C, D). This organism was not enclosed within a pansporoblast membrane as were similar pansporoblasts observed in other Microsporidian species (Vey and Vago, 1973; Vavra, 1965). Furthermore, examination of serial sections of this dividing sporont revealed that the nucleus of each sporoblast is not double but single with a deep cleft.

Other cells of marked electron-density were noted frequently (Pl. 3F). Typically they were spherical or slightly elongated and about 3-4 μm in diameter. Most showed two large nuclei separated by compressed nuclear membranes and a rough endoplasmic reticulum organized into concentric rings around

the nucleus. The significance of this phase is not known.

DISCUSSION

Features of the disease reported here are in agreement with previous accounts of Thelohania contejeani (Henneguy and Thelohan, 1892; Kudo, 1924; Vey and Vago, 1973). Principle diagnostic features are the octosporous pansporoblast and the dimensions of the living spore.

Henneguy and Thelohan (1892) and Schäperclaus (1954) have both indicated that T. contejeani had a marked effect upon the crayfish populations studied, with a 30% infection rate recorded by the latter worker. Recent publications, however, all report low infection rates (Vey and Vago, 1973; Sumari and Westman, 1969). In Northumberland less than 10% of all crayfish caught (over 400) were infected, even though the disease has been present in these same streams for at least 5 years. The reasons for the low infection rate of the local crayfish population are not known, but they may be associated with the physical nature of the waterways and perhaps with the size of the crayfish population. Clearly, the Microsporidian does not present the same problems as the crayfish plague Aphanomyces astaci (Svärdson, 1965).

The disease is probably disseminated upon breakdown of the dead host crayfish. Hence the most effective counter-measure is to destroy all infected individuals before their spores

are dispersed. Cannibalism of infected tissues may also be an important source of infection. It has been suggested that the polar filament is everted in the host gut (Schäperclaus, 1954; Voronin, 1971) and the sporoplasm 'germinates' into the gut epithelium and thence to the muscle tissues. The wide distribution of the parasite within the body of an infected animal suggests the presence of a dispersive phase acting through the haemolymph. Examination of the haemolymph of infected crayfish did reveal spores, but it is not known if these are the true dispersive phase or are present merely as a result of muscle fibre lysis and release of the spores. The end result of infection is the massive breakdown of host muscle tissue and a consequent loss of muscular power, as confirmed myographically by Henneguy and Thelohan (1892). Presumably this leads to the virtual immobility of the host and hence death. The parasite may have few other direct effects on the host, since infected crayfish were observed to moult normally and the gonads were often well formed. The time course of the disease is not known, though several infected crayfish have been maintained in the laboratory for several months without any dramatic deterioration in their condition.

The presence of parasitized and unparasitized muscle fibres lying adjacent to each other (Pl. 1B) suggests that the parasite is slow to invade every muscle fibre (due perhaps

to the thick collagen sheath), but once entry has been effected muscle lysis and formation of a spore mass is rapid.

Ultrastructurally, the schizonts, sporonts, pansporoblasts and spores of T. contejeani are similar to other Microsporidian species (Huger, 1960; Kudo and Daniels, 1963; Jensen and Wellings, 1972; Sprague, Vernick and Lloyd, 1968; Sprague and Vernick, 1968, 1969; Vavra, 1965; Vey and Vago, 1973). The double appearance of the nuclear apparatus is thought to be characteristic of all stages of the parasite. However, the dividing sporont nucleus has been shown to be single with a deep cleft and it must be considered that this form of the nuclear apparatus may be common to all stages of the life-cycle. Vey and Vago (1973) have indicated that the spore nucleus is single but has become elongated and curved to form a U-shaped structure. The presence of two apparently separate nuclear spindles (Pl. 3A and Sprague and Vernick, 1969) in adjacent nuclei suggests that there may be two sets of chromosomes, contained within a single nuclear membrane, a view originally proposed by Debaisieux (1920). Ultrastructural observations by other workers claiming to show fusion of nuclei (Sprague and Vernick, 1968), binuclear and uninuclear schizonts should be re-examined since they all may be different sections of the same nuclear configuration.

It is proposed that the sequence of events during spore

formation in T. contejeani may be as follows. First, the repeated division of the sporont nucleus to yield nuclei, each of which has a characteristic double appearance. This octanuclear sporont undergoes cytokinesis to form eight sporoblasts (Pl. 4C, D) which may or may not be enclosed within a pansporoblast membrane. Each sporoblast differentiates to form a spore (Pl. 4A, B) which ultimately matures and develops a spore capsule (Pl. 2A, B).

The ultrastructural observations reported here agree in most respects with those reported by Vey and Vago (1973), particularly in details of spore size and structure. However, some differences are apparent, particularly concerning the pansporoblast. The mass of tubular structures formed within the pansporoblast, but external to the sporoblasts and spores, does not resemble the moniliform structures shown by Vey and Vago (1973). This disparty is probably not due to fixation artefact since Vey and Vago (1973) used similar techniques as used in this study (A. Vey, personal communication).

In a few instances, the pansporoblast membrane persists so that mature spores are enclosed by it, whilst in other cases dividing sporonts, late sporoblasts and immature spores are not so enclosed. Vey and Vago (1973) did not comment on this feature, and this may be a clear difference between the parasite studied by us and Vey and Vago (1973). The persistence of the pansporoblast membrane may be a useful

taxonomic character since in T. petrolisthis and T. maenadis, both of which parasitize marine decapods, it is most persistent (V. Sprague, personal communication). In this respect the Microsporidian reported here resembles T. cambari which is parasitic in an American crayfish (Cambarus bartoni), for Sprague (1950) reports that the sporont (pansporoblast) membrane is non-persistent, and the spores always become separated as they approach maturity.

Fig. 1:

Diagram of protozoan stages from a muscle homogenate smear observed under the light microscope. For details refer to text. All stages observed under phase contrast except B, C and I, which were stained with Giemsa. Scale for A-K represents 4 μm and for L represents 10 μm.

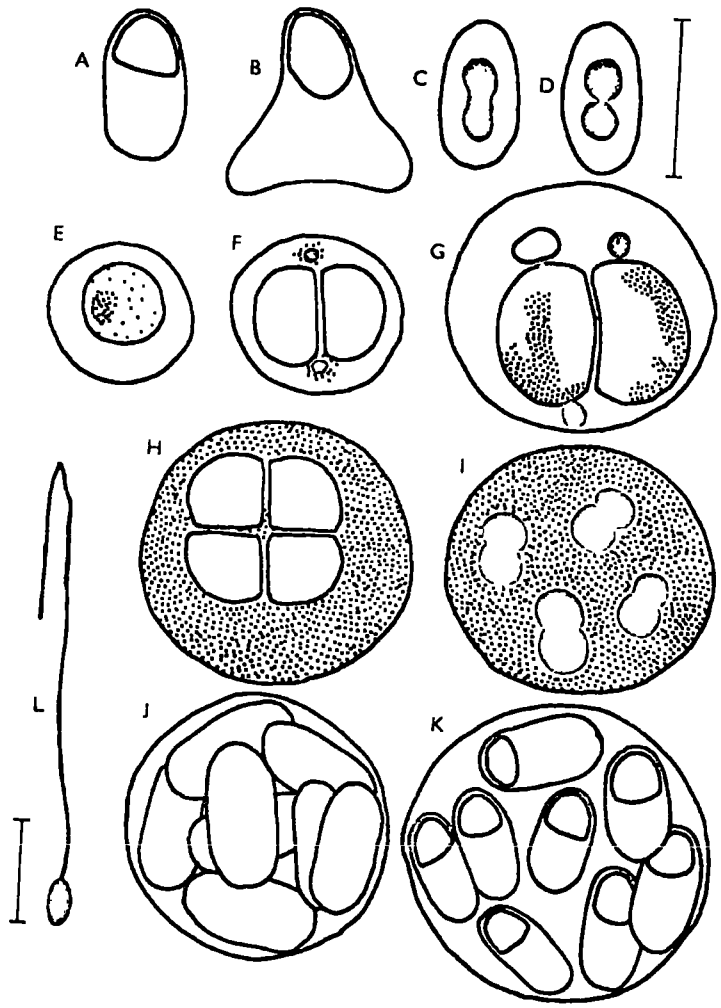
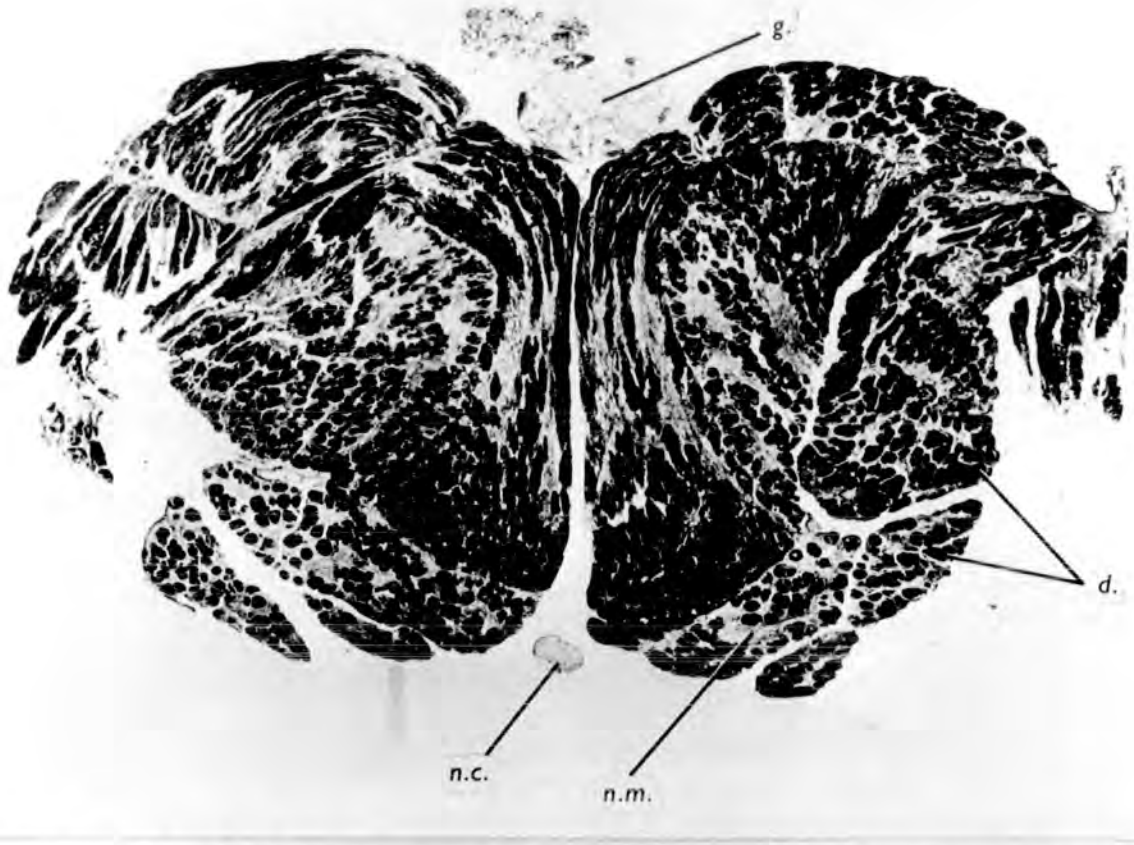

A
B
C
D
E
F
G
H
I
J
K
L

Plate 1:

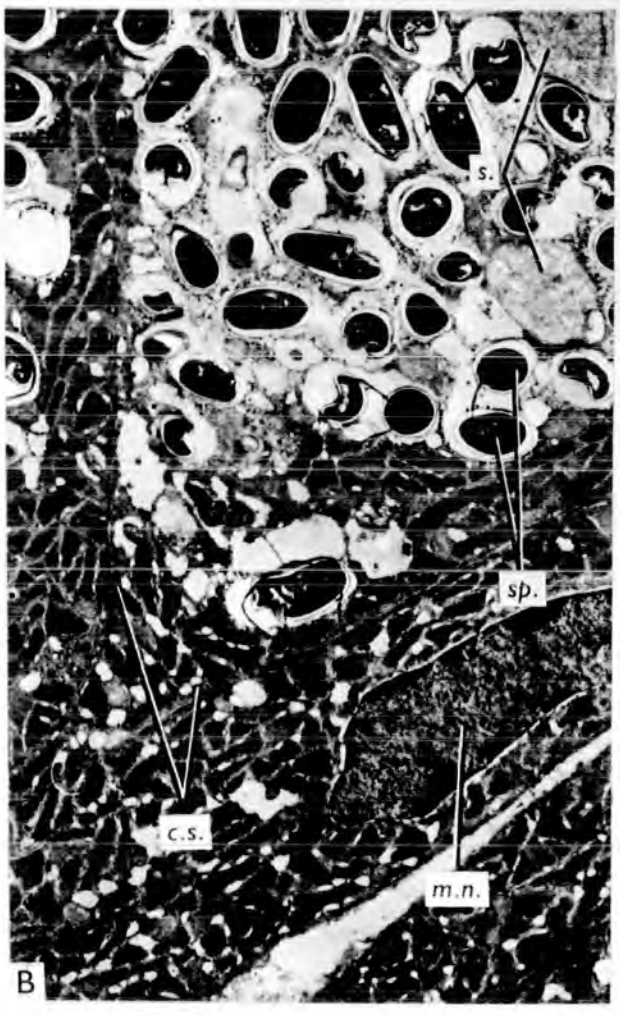
A. Transverse section through the entire abdominal flexor muscle preparation of a diseased crayfish. Infected muscle fibres (d.) appear as dense black masses. Uninfected muscle fibres retain a grey striated appearance (n.m.). (Heidenhain's iron haematoxylin.) (x 12.)

B. Electronmicrograph of infected muscle tissue showing spore masses enclosed within a muscle fibre. Adjacent muscle fibres are often unparasitized and ultrastructurally normal. (x 3200.)

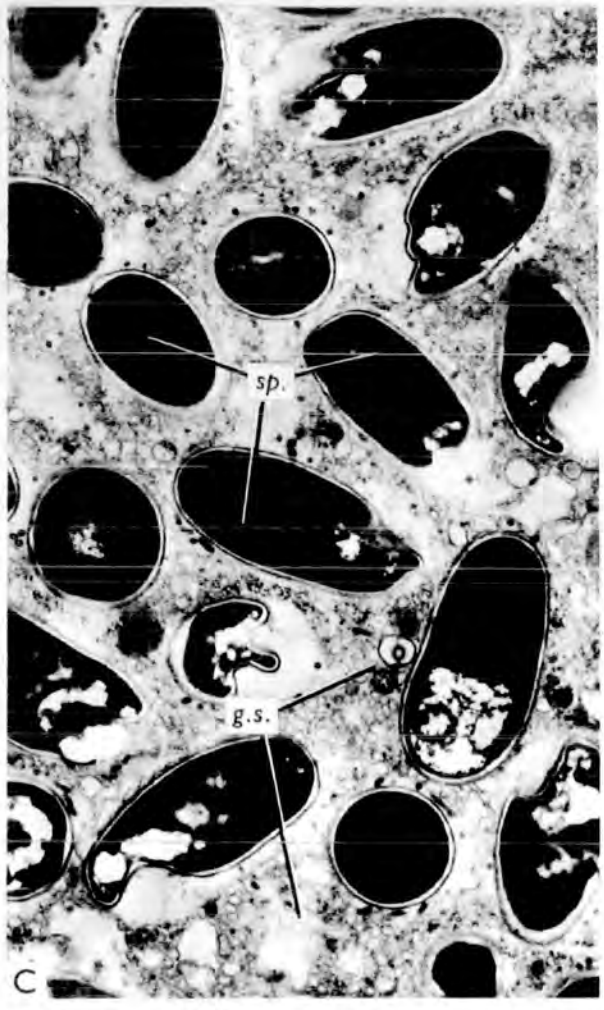
C. Detail of the spore mass. Electron-dense spores (sp.) are surrounded by an interstitial ground substance composed of granules and vesicles (g.s.). (x 8000.)



A



B



C

Plate 2:

A. A typical spore. For details see text. (x 25000.)

B. Electronmicrograph of a mature spore showing a double nuclear apparatus. (x 27000.)

C. High-power electronmicrograph of a spore in the region of the coiled polar filament. The spore is surrounded by a crenelated membrane (c.m.), a bilaminate spore capsule (sp.c.) and a spore membrane (sp.m.). The polar filament is membrane-bound (c.p.f.) and is coiled around the posterior vacuole (p.v.). (x 80000.)

D. Detail of the tubular structures (t.) found within a mature pansporoblast. These structures may be seen in transverse and longitudinal section. (x 36000).

E. High-power electronmicrograph of the anterior region of the spore, showing the polar sac (p.s.), swollen base of the polar filament (b.f.) and the laminated polaroplast (p.t.). (Uranyl acetate only.) (x 50000.)

F. Electronmicrograph of a typical schizont possessing a characteristic double nucleus (n.) and a well developed endoplasmic reticulum (e.r.). (x 10000).

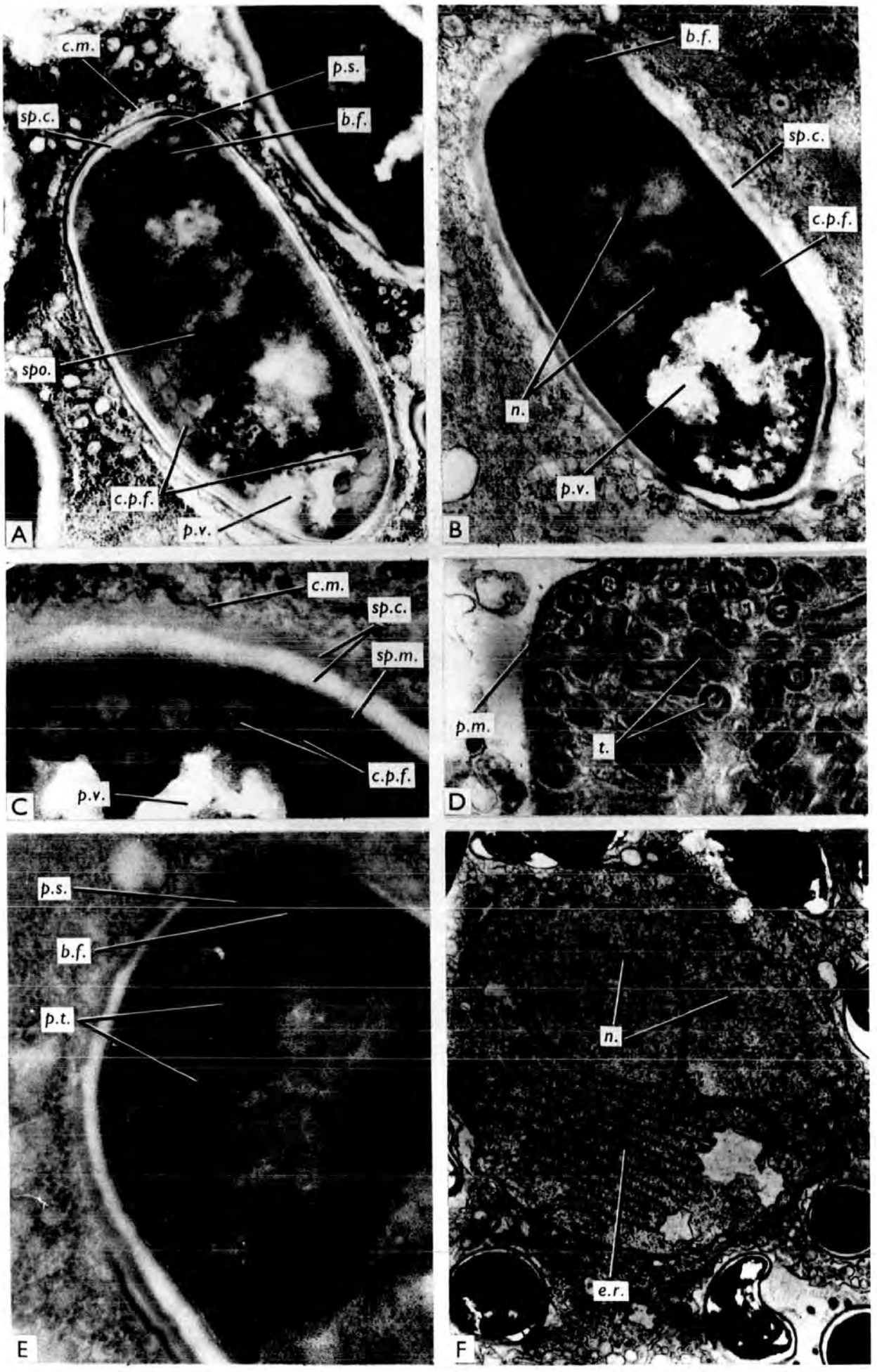

c.m.
p.s.
sp.c.
b.f.
spo.
c.p.f.
p.v.
A
b.f.
sp.c.
c.p.f.
n.
p.v.
B
c.m.
sp.c.
sp.m.
c.p.f.
p.v.
C
p.m.
t.
D
p.s.
b.f.
p.t.
E
n.
e.r.
F

Plate 3:

A. Electronmicrograph of a diplocaryon undergoing division. The spindle apparatus (s.a.) and centriole (c.) are well developed. (x 20000.)

B. Electronmicrograph of a simple schizont. It appears uninucleate in section. Note the disorganized and poorly developed endoplasmic reticulum. (x 10000.)

C. Electronmicrograph of a schizont containing a mass of small, membrane-bound vesicles (v.) within a distinct plasmalemma. The nucleus shows evidence of spindle and centriole formation. (x 12300.)

D. Electronmicrograph of a presumed sporont with the nuclei arranged in two pairs. The endoplasmic reticulum is not developed, but there are numerous large vacuoles, probably artefactual. (x 10000.)

E. Electronmicrograph of a pansporoblast showing five mature spores. Other unidentified tubular (t.) structures and electron-dense granules are apparent within a distinct pansporoblast membrane (p.m.). (x 8000.)

F. Electronmicrograph of a small 'diplocaryon' of marked electron-density. Two nucleoli are evident (nu.). The endoplasmic reticulum is organized into concentric rings around the nuclear apparatus. (x 20000.)

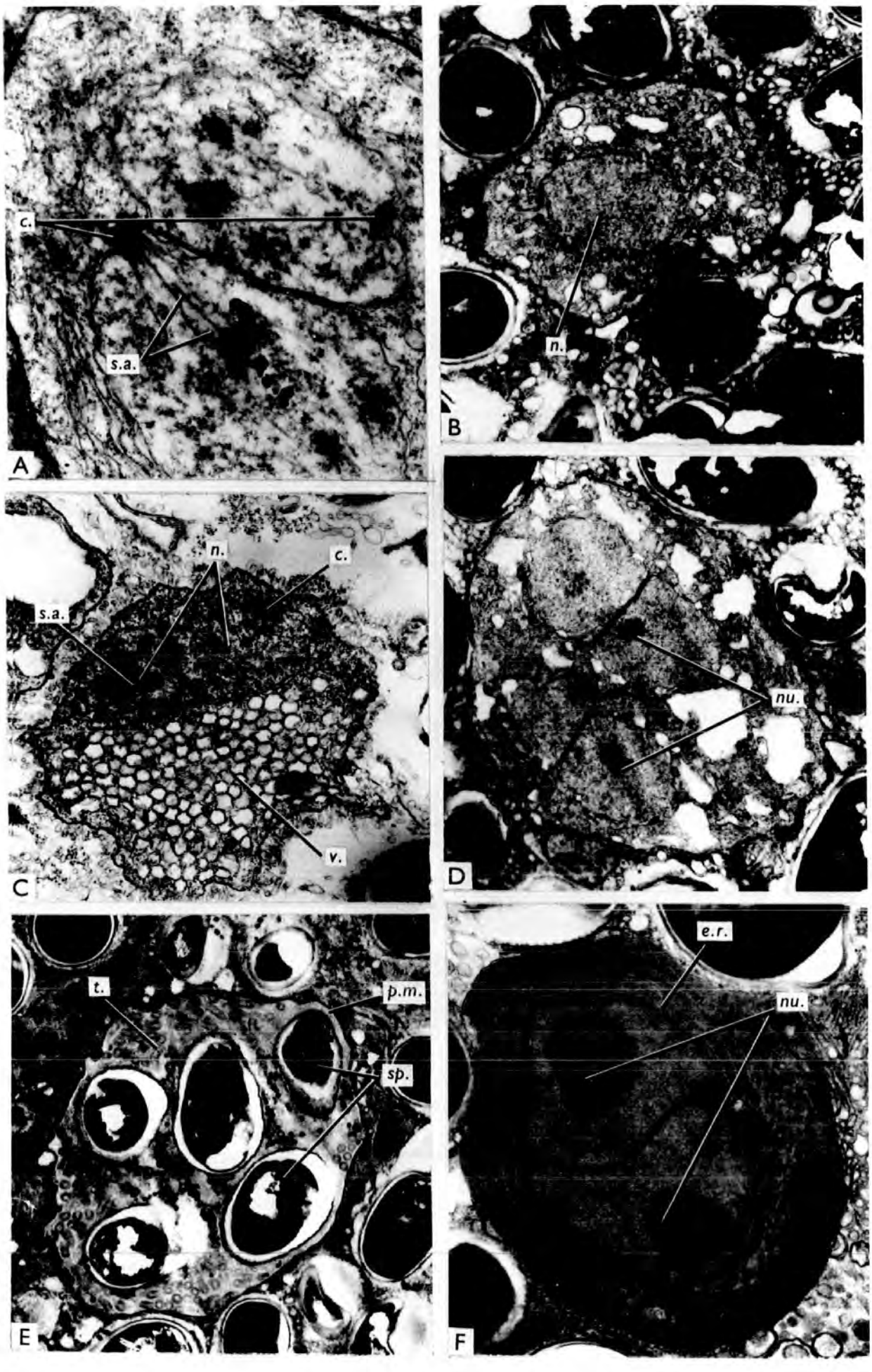
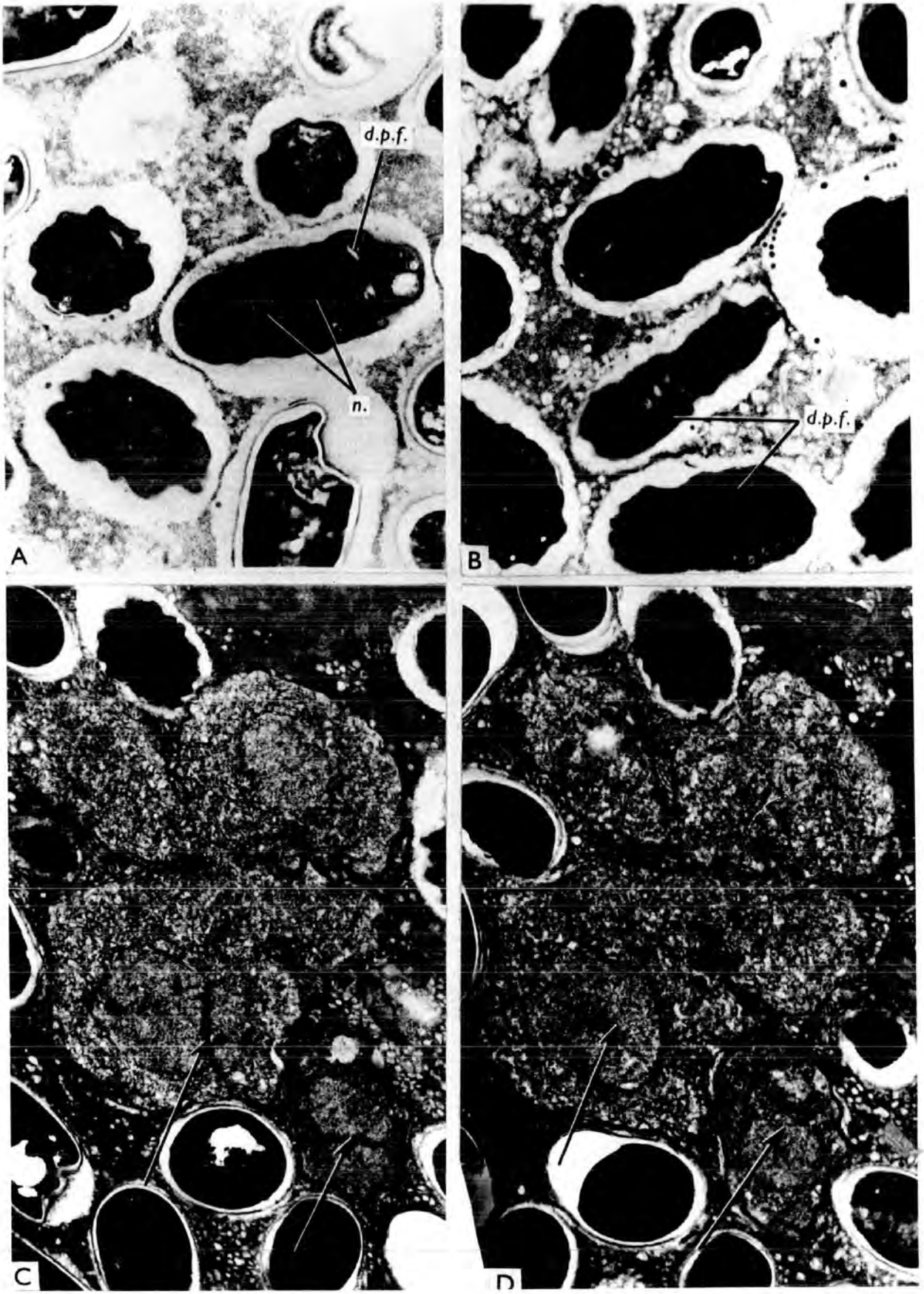

c.
s.a.
A
n.
B
n.
c.
s.a.
C
nu.
D
t.
p.m.
sp.
E
e.r.
nu.
F

Plate 4:

A. Electronmicrograph of presumed late sporoblast. A double nucleus is obvious and is surrounded by an organized endoplasmic reticulum. The polar filament has begun development (d.p.f.) at the anterior pole. The sporoblasts have shrunk during fixation since they do not possess a spore capsule. (x 10000.)

B. Electronmicrograph of immature spores. They possess a fully developed polar filament and in some spores a double nucleus is visible. The spores have shrunk away from the surrounding cytoplasm during fixation. (x 10000.)

C,D. Electronmicrographs of serial sections of a sporont undergoing division. In C one of the developing sporoblasts has a double nucleus (arrow) but in the subsequent section (D) this nucleus is single. No pansporoblast membrane is evident. The adjacent sporoblast also shows a similar configuration (arrow). (x 8400.)


d.p.f.
n.
A
d.p.f.
B
C
D