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HEAT DEATH AND THE DEVELOPMENT OF THERMOTOLERANCE IN THE BLOW FLY *CALLIPHORA VICINA*: A STUDY OF FLIGHT MUSCLE MITOCHONDRIAL FUNCTION.

by

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A thesis submitted in candidature for the degree of Doctor of Philosophy

Department of Biological Sciences
The University of Durham

(Graduate Society)

1996

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10 MAR 1997

Thesis
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"In memory of my mother who passed away earlier"

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Declaration:

I hereby declare that the work presented in this document is based on research carried out by me, and that this document has not been presented anywhere else for a degree. Part of this document has been published in the Journal of Experimental Biology (El-Wadawi & Bowler, 1996).

ABSTRACT

The LD_{50} of 10-day-old blowflies differed significantly in two different stocks, and were found to be 38.12 ± 0.07°C for the Durham stock and 40.8 ± 0.18°C for the Cambridge stock.

A transitory increase in heat resistance occurred following the exposure of adult blowflies to a sublethal heat shock. This thermotolerance was apparent 1h after the application of heat shock, was maximal 2-3 h later and had disappeared after 6 h.

Oxidative phosphorylation by flight muscle mitochondria from non-thermotolerant control flies was impaired by an LD_{50} dose *in vivo*. Respiration using glycerol-3-phosphate was more heat sensitive than that with pyruvate plus proline. State III respiration was markedly inhibited, acceptor control (RCI) was lost with (G 3P) as substrate and so ADP:O ratios were not measurable; whereas with pyruvate + proline as substrates, although State III respiration was inhibited by 50% and acceptor control was significantly reduced, ADP:O remained measurable. Uncoupling of oxidative phosphorylation was obvious only with pyruvate + proline where State IV was significantly increased.

The development of thermotolerance protected oxidative phosphorylation against heat damage. With G-3-P respiration State III was largely restored and acceptor control was not significantly different from controls, but ADP:O remained lower. With pyruvate + proline as substrates State III respiration was inhibited, but State IV was also lower without evidence of uncoupling of oxidative phosphorylation. Acceptor control was restored to control levels but ADP:O values were lower. The lower ADP:O ratios indicate some impairment of mitochondrial function occurred.

The effect of experimental temperature *in vitro* on respiratory performance of mitochondria from non-pretreated control and thermotolerant LD_{50} flies was also determined between 19 and 39°C. State III respiration was markedly temperature-dependent in mitochondria **from** non-pretreated control flies with both substrates; it was maximal at 24-29°C and fell progressively at higher measuring temperatures. In mitochondria from thermotolerant flies, State III respiration was less temperature

dependent with both substrates, but this effect was more marked for G-3-P. The effect of experimental temperature on State IV respiration was similar in mitochondria from non-pretreated control and thermotolerant LD_{50} flies with the same substrate, but differed between the two substrates. With G 3P as substrate, respiration rate rose with temperature with a Q_{10} of approximately 1.5; however, with pyruvate + proline as substrate, the trend was for respiration rate to fall as experimental temperature rose.

Differences in the temperature sensitivities of mitochondria from control and thermotolerant flies, in terms of acceptor control, were found. Using G-3-P, acceptor control was lost in mitochondria from control flies above 29°C, but was still measurable at 34°C in mitochondria from thermotolerant flies. With pyruvate + proline as substrate acceptor control was demonstrable in mitochondria from both non-pre-treated control and thermotolerant flies at all experimental temperatures.

The thermal sensitivities of the respiratory complexes were studied using the inhibitors rotenone and antimycin A. In mitochondria from LD_{50} treated control flies respiration uncoupled with FCCP was not restored to State II levels. However, in LD_{50} treated mitochondria from thermotolerant flies respiration uncoupled with FCCP was not different from State III respiration. These data suggest that the reduction in State III respiration after heating is owing to an inhibition of oxidation rather than phosphorylation. Complex I, NADH coenzyme Q reductase, was shown to be the most temperature sensitive of the respiratory complexes.

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GLOSSARY

ADP	Adenosine-5'-diphosphate
ADP:O	ADP:O ratio (number of molecules of ADP esterified per atom of oxygen consumed)
ATP	Adenosine-5'-triphosphate
ATPase	Adenosin-5'-triphosphotase (E C 3 . 6 .3 .1.)
BSA	Bovine serum albumin (fraction V)
DNP	2,4 dinitrophenol
EDTA	Ethylenediamine tetra-acetate (sodium salt)
G 3P	Glycerol 3-phosphate
Km	Michaelis constant
LD_{50}	Experimental heat treatment causing 50% mortality in a sample animals.
NAD^+	Nicotinamide-adenine dinucleotide(oxidised form)
NADH	Nicotinamide-adenine dinucleotide (reduced form)
Q_{10}	Temperature coefficient
QO_2	Oxygen uptake expressed as µg atoms oxygen per mg per hour
RCI	Respiratory control index (acceptor control)
TRIS	Tris (hydroxymethyl) aminomethane
V_{max}	Maximal reaction velocity

CHAPTER I

GENERAL INTRODUCTION

The influence of temperature on animals has been the subject of many reviews (see Prosser and Heath, 1991; Precht, Christophersen and Hensel, 1973; Cossins and Bowler, 1987). Heat injury is experienced by plant and animal cells, as well as by microorganisms, at temperatures that are elevated only slightly above their normal range. Heat is peculiar, amongst possible physical and chemical insults that organisms may experience, in that it is all pervasive (Bowler, 1987).

The normal temperature range of a species is fairly well-defined, and is that within which the organism can function normally. This range depends upon evolutionary thermal experience and is genetically determined (Prosser, 1973). Exactly what causes death at the limits of this range remains still in doubt, but it is likely that the causes of cold death and heat death will differ. Indeed, Bursell (1964) has argued that since the point at which death occurs varies between species over so wide a range, it is unlikely that the cause of death could be the same or could be simple.

The damaging effects of high temperatures on organisms are dose dependent and so it is not possible to quote a single lethal temperature for an animal without stipulating the exposure period. A high temperature that is tolerated for a few minutes by an animal may become lethal over a longer period. Even within the genetically set limits the actual temperatures tolerated by an animal may be influenced by such factors as developmental stage and history, age and sex. Thermal tolerance is also dependent on previous thermal history, acclimation and acclimatisation to temperature resulting in phenotypic changes in thermal resistance (Cossins and Bowler, 1987). The dependency

of the heat death point of a population of animals on acclimation was called resistance adaptation (acclimation) by Precht *et al.,* (1973). The mechanisms conferring resistance acclimation are not understood, but it is a phenomenon demonstrated in species from all phyla of the Animal Kingdom. It is therefore considered to be an important adaptive strategy in the responses animals make to changes in environmental temperature (Cossins and Bowler, 1987).

It is usual that heat death points remain unchanged as long as culture temperature is also unchanged. However, Davison (1969), working with *Calliphora erythrocephala*, confirmed earlier studies on other holometabolous insect species that a dramatic fall occurred in heat death point during the early adult period even though culture temperature was unaltered. This phenomenon was first described by Burnett (1957) in *Glossina.* Later Baldwin (1954) reported a similar decline in tolerance in young chalcids *Dahlbominus fuscipennis*, but attributed it to the fact that the animals were not fed during the period of the experiment. Hollingsworth and Bowler (1965, 1966) showed that this phenomenon also occurred in *Drosophila subobscura*, and suggested that this loss of heat tolerance was a developmental change. They argued that the high tolerance of the newly emerged adult was carried over from the immobile pupal stage, but was lost because it was unnecessary in the mobile adult stadium.

The decline in early adult blowflies is markedly temperature dependent (Bowler, 1981), which suggests the change in tolerance is dependent on the metabolism of the blowfly. For example, Bowler, (1981) reported on the LD_{50} points, 5 and 10 days after emergence, when flies were transferred to a temperature between 5 and 34°C. At 5°C no decline in LD_{50} had occurred after 10 days, but as the temperature of transfer rose the rate of decline increased, to reach a maximum at 29°C. The expected acclimation effects on LD_{50} did not occur until the temperature transfer rose to 32° and 34°C, only

at these higher rearing temperatures is the developmental decline in LD_{50} overcome by acclimation.

These reports, from a variety of species of endopterygote insects, suggest these changes in physiological resistance in the early adult may be a general phenomenon. These developmental changes in the thermal tolerance of blowflies have been taken into consideration in the planning of the present experimental work.

Much of previous literature describes the establishment of heat death points and factors affecting them, but few studies have concerned the mechanisms involved in heat death. As a consequence of the wide variety of these and other studies, much confusion occurs in the literature as to the causes of heat death . The problem can be stated as follows: as heat injury is a universal feature throughout the Animal and Plant Kingdoms, as well as in microorganisms, are there fundamental mechanisms involved in heat injury that are common to all organisms ?

The precise mechanisms of heat death are not understood and the sequence of physiological events which leads to death when animals are exposed to a high temperature is still poorly known. The underlying causes of heat death have been studied for a long time and in a variety of organisms, yet the literature has produced a number of theories, some mutually exclusive, as to the causes of heat death.

The general problem of cause and effect makes the identification of the mechanisms of heat death difficult, and the separation of those lesions which initiate heat death from the secondary and tertiary consequences of those lesions is often unclear, however, as heat injury is experienced by all organisms (and cells) it is reasonable to consider that the primary lesion may be common (Bowler, 1987).

The influence of temperature in ectotherms has stimulated a multitude of studies on the mechanism of adjustment to changing thermal environments (Prosser, 1973;

Precht *et al* 1973; Hutchison and Maness, 1979). White and Somero (1982) have pointed out that while both resistance and capacity adaptations of ectotherms have been extensively studied under steady state acclimation conditions, little is known about either rapid changes in their physiology, such as may occur during diurnal temperature fluctuations, or transition phases that occur during the course of acclimation. Thermal tolerance and adjustment of thermal tolerance are of interest because survival under changing thermal conditions is thought to be a prime determinant of the presence or absence of a species in a particular geographical area or habitat (Brett, 1956; Hutchison, 1961; 1976; Brattstrom, 1968; Fry, 1971; Spotila, 1972; Hutchison and Maness, 1979). Ectotherms respond to environmental temperature changes with a variety of behavioural and/or physiological responses.

Resistance acclimation and thermal hardening are two such physiological responses (Hutchison and Maness, 1979). Although both involve compensatory shifts in temperature tolerance, the temporal aspects of the two phenomenon are quite distinct (Hutchison and Maness, 1979). Acclimation is a slow but long lasting response to long term temperature change. It typically occurs within the range of temperatures normally experienced by the species, and is reversible. Thermal hardening is rapid requiring only minutes to a few hours for completion. It has been proposed that hardening is a rapid transitory response following brief exposure to near lethal temperatures (Precht *et al.,* 1973; Hutchison, 1980); and multiple exposures do not increase thermal hardening over the level induced by a single shock (Hutchison and Maness, 1979). More knowledge concerning the mechanism of hardening may lead to increased insight into mechanisms of heat injury.

Clear evidence now exists, for a variety of ectothermal vertebrates, for heat hardening under field conditions. The initial evidence came from the desert pupfish

Cyprinodon macularis (Lowe and Heath, 1969). These fish experience diurnal temperatures of 40-41°C that are close to their thermal limit. Maness and Hutchison (1980) also report that periods of peak heat hardening coincided with the periods of higher environmental heating in a number of fish and amphibians. A similar findings was reported for two species of lungless salamanders by Rutledge, Spotila and Easton (1987). Hutchison and Maness (1979) have clearly drawn a distinction between resistance acclimation and heat hardening, based on the very different time-courses of the two phenomena.

Cold hardening is induced by exposure to low temperature and aids survival against exposure at lower temperatures. Recently a "rapid-cold hardening" response has been reported in a number of insects, particularly the flesh fly *Sarcophaga crassipalpis*, which confers protection against injury due to "cold shock" at temperatures above the supercooling point (Lee, Chen Mecham and Denlinger, 1987; Chen, Lee and Denlinger, 1991). For instance, when pharate adult pupae of *Sarcophaga crassipalpis* were transferred from 25 to -10°C for 2 h there was 100% mortality, however, when pharate adult pupae were transferred from 25 to 0°C for 2 h prior to exposure to -10°C there was >91% survival (Chen, Denlinger and Lee, 1987). A similar rapid increase in cold tolerance has also been investigated in other insects including the house fly *Musca domestica* (Coulson and Bale, 1990), *Drosophila melanogaster* (Czojka and Lee, 1990), Gypsy moth, *Lymantria dispar* (Denlinger, Lee, Yocum and Kucal, 1992). Direct transfer of the various life cycle stages of these insects from their culture temperature to a particular sub-zero temperature causes "cold-shock" mortality. In contrast, if the insects are pre-exposed to a higher subzero temperature first most survive the subzero exposure. Thus whilst prior exposure to sublethal high or low temperatures can cause a rapid biological response that affords

protection against subsequent heat or cold injury, the nature of these responses remains largely undefined.

The physiological basis of heat hardening is obscure. It has been demonstrated not only at the organism level, but also in isolated tissues and in cultured cells of both invertebrates and vertebrates. Heat hardening in response to a sudden heating, is also referred to as thermotolerance. This phenomenon (thermotolerance) originally reported by Gerner and Schneider (1975) and Henle and Leeper (1976) has been substantiated by many investigators (Landry, Lamarche and Chretien, 1987; McAlister and Finkelstein, 1980; Mivechi and Li, 1985; Subjeck, Sciandra and Johnson, 1982). The various studies in this field of acquired thermotolerance have been reviewed by Hahn and Li (1990) and Hightower (1991). The biochemical mechanisms involved in thermotolerance have been subject to intense investigation.

It is significant that in a variety of cells, ranging from yeast to mammalian cell lines, a family of proteins is synthesised in response to sublethal heat shock, these are known as the heat shock proteins (HSPs). This induction is called the heat shock response, and is an ubiquitous phenomenon, from bacteria to man. These proteins (HSPs) which are well conserved in evolution are generally grouped according to their molecular weight: The HSPs 83-90KD family, the HSPs 68-70 kD family, and the small HSPs 30-40 KD molecular weight whose molecular weights (MWs) are variable and which show less conservation in evolution (Schlesinger, 1990). Some of the members of these families are constitutively expressed and their production is simply 'up-regulated' during stress. Other members are not normally present and are produced only as a result of the induction of gene expression producing mRNAs which are then translated.

Although their original discovery by Ritossa (1966) in *Drosophila* passed almost unnoticed, HSPs response has been vigorously investigated by numerous laboratories during the past 15 years. HSPs were purified from *Drosophila* by Tissieres *et al* (1974). For about 20 years, this selective induction of proteins by heat-shock was thought to be unique to the fly. In 1978, however, an analogous response in avian and mammalian tissue culture cells to heat shock was discovered (e.g. Kelley and Schlesinger, 1978) as well as in *E.coli* (Neidhart, Lemaeax, Herendeen and Bloch, 1978).

The function and significance of these heat shock proteins is not fully understood, but they appear to be involved in a general mechanism that helps organisms to survive physiological insult, and, depending on the experimental system under study, various HSPs have been implicated as contributing in the acquisition, maintenance and decay of thermotolerance (Lee and Dewey, 1987; 1988; Laszlo, 1988). This is mainly based on the close temporal correlation between the accumulation of heat shock proteins and the development of thermotolerance (Hahn and Li, 1990; Hightower, 1991; McLennan and Miller, 1990; Parsell and Lindquist, 1993; Bosch *et al.,* 1988; Landry *et al.,* 1982). However, the relative importance of different heat shock proteins in thermotolerance varies between organisms. Stephanou & Alahiotis (1982), indicated that HSPs could be a major target of temperature-induced selection. If this is true then HSPs can be considered to be as another important molecular mechanism through which insects respond to temperature.

Studies seeking to correlate the induction and synthesis of HSPs with the development of thermotolerance have largely been carried out on cells in culture. That HSPs are connected with the acquisition, maintenance and decay of thermotolerance in mammalian cells was indicated from experiments of Landry *et al.*, (1982), in which

synthesis and degradation of heat shock proteins was followed during development and decay of thermotolerance. In addition the level of certain HSPs, particularly the HSP70 in murine tumours (Li and Mak, 1985), murine embryos (Muller, Li, and Goldestein, 1985), *Xenopus* embryos (Heikkila, Kloc, Bury, Schutz and Browder, 1985) and Chinese hamster fibroblasts (Li and Werb, 1982) have been reported to correlate with thermotolerance.

There have been, however, some investigations which contradict a direct correlation between the accumulation of heat shock proteins and the development of thermotolerance. For example, tolerance has been induced in the absence of HSPs synthesis (Carper, Duffy and Gerner, 1987); furthermore, Smith and Yaffe (1991) showed that HSPs induction is not required for thermotolerance acquisition in yeast. Moreover, some studies have failed to show a correlation either between specific HSPs or general protein synthesis and thermotolerance (Loomis and Wheeler, 1980; Hall, 1983; Widelitz, Magun and Gerner, 1986). HSPs may therefore not be the only protective agents in tolerance (Laszlo, 1988; Boon-Niermeijer, Tyl and Van de Scheur, 1986).

Heat shock proteins are not only important in situations of heat shock (and other stresses), where they were detected first, and where their name originates, but they also appear to be essential for cell survival in normal situations. HSPs and their cognates have been implicated in various roles, and evidence has emerged over the last few years, that HSPs have been shown to play crucial roles in intracellular protein metabolism, dynamics and also one of the most prominent roles is in protein folding and degradation (Dice, Chiang, Terkecky and Olson, 1991). This has led to the concept of HSPs as chaperones. Furthermore, ubiquitin, a member of a HSPs family, binds to unfolded proteins which will then be degraded during protein turnover in cells

(Mayer, Arnold, Laszlo, London and Lowe, 1991), as it was proposed that HSP70 unfolds partially folded polypeptides, so that they can be translocated through membrane pores. HSP60 also facilitate the proper folding and assembly of the newly-transported proteins in mitochondria (Horwich, Hartl, and Cheng, 1991). Consistent with this role are data showing that an HSP70 -like protein can bind to and, in the presence of ATP, dissociate protein complexes (Schlesinger, 1990). They also participate in the degradation of proteins. This activity serves to protect the cell, for not only is proteolytic turnover an essential activity in normal cell, but accumulation of denatured protein could be toxic (Schlesinger, 1990).

The likely involvement of HSPs in protein metabolism is interesting because historically, heat death has been attributed to protein coagulation and enzyme inactivation. For example, Ushakov, (1964) reported on the similarity in Arrhenius values (E_a) for cellular heat death and protein denaturation, and concluded that heat injury resulted from the latter effect. However, as most enzymes tested had a greater thermostability than the cells or organism from which they were extracted, Ushakov, (1964) suggested that heat sensitivity of cells is determined by their least resistant proteins. Rosenberg, Kemeny, Switzer and Hamilton (1971) have produced evidence on thermodynamic grounds, that heat death in yeast, bacteria and viruses was due to denaturation of proteins. However, Read (1967) gave a clear lead in this field when he argued considerable caution in relating heat resistance of an organism to the thermostability of its proteins, unless it could be demonstrated that the temperatures at which protein function fails, closely coincides with those at which metabolism fails. In very few cases has this criterion been met. The best documented work has been in myosin ATPase in lizard (Licht, 1964), on an amylase from a thermophilic bacterium (Manning and Campbell, 1961) and on Mg^{2+} dependent ATPase from crayfish muscle

sarcolemma (Bowler and Duncan, 1967; Gladwell, Bowler and Duncan, 1976). More recently the Ca^{2+} Mg^{2+} ATPase from crayfish muscle sarcoplasmic membranes has been shown to be heat sensitive at the same temperature range as caused heat death; (Cossins and Bowler 1976).

Hochachka and Somero (1973) remarked that protein (enzyme) function may be affected at temperatures well below denaturing temperatures, e.g. as a result of temperature changes in enzyme-substrate affinity. In allosteric enzymes this may also apply to temperature dependent changes in the sensitivity of the enzyme to its allosteric effector. Thus, heat death may result from temperature induced reversible inhibition of activity, through changes in tertiary and quaternary structure of a critical enzyme(s). The hierarchical sequence, referred to earlier, indicates that the sensitivity to heat in animals followed the pattern whole organism-organ system- cell - organelle-enzyme (Ushakov, 1964), that is to say, the organism is found to be the most sensitive to heat, and the functioning of an organ is less sensitive, and that of cells are less sensitive still, whilst individuals proteins are the least sensitive. This sequence indicates that heat death is a complex phenomenon and not easily explained simply in terms of protein stability.

Although in recent times research in this field has focused on heat injury to cells, this approach avoids the complicating factors associated with physiological integration as a factor in organism death. In spite of an extensive search for the exact target(s) for hyperthermic cell death has been carried out, the critical target (primary lesion) and the molecular mechanism(s) of heat induced cell killing remain unsolved. A further complicating possibility in the search for the primary damage is that its perturbation by heat may last only for the duration of the heat application, but that resulting secondary and tertiary damage may be the damage observed and these may be cell or organism

specific. A schematic representation shown in Figure 1.1, by Bowler (1987), suggested that primarily heat damage (lesion) which need not be, (or become) irreversible, caused a cascade of secondary and tertiary "knock-on" effects which, in a time-dependent manner, will cause irreversible injury and lead to death. Recently Jung (1991) suggested, from data analysis of survival curves, using mathematical models, that cellular heat damage is a two step process, where primary damage is converted to permanent damage in a time-dependent fashion. This is in agreement with the model for cellular heat sensitivity proposed by Bowler (1987) mentioned above.

Evidence has been presented that the nucleus is affected at hyperthermic temperatures, in particular, Warters and Roti Roti (1982) observed that the condensation of material into the perinuclear region was a prominent feature which was accompanied by an increase vessiculation of the nuclear membrane (Heine, Severak, Kondratick and Bonar, 1971; Welch and Suhan, 1985; Warters, Yasui, Sharma and Roti Roti, 1986). The nucleolus was reported to be a very heat sensitive organelle, undergoing marked changes (Simard and Bernard, (1967). The increased nuclear protein content observed following hyperthermia was a large and rapid effect, that correlated with heat-induced cell killing (Roti Roti, Henle and Winward, 1979).The presence of excess nuclear proteins may be involved in inhibition of DNA replication (Wong & Dewey, 1982; Laszlo, 1992). Furthermore, Dewey, Harpwood, Sapareto and Gerweck (1971) have suggested that chromosome aberrations are involved in heat cell death.

The evidence so far also indicates that heat i) induced direct alterations in cytoskeletal components, interfering with their ability to self-assemble (Alberts *et al.*, 1984; Welch and Suhan, 1985; Coss, Dewey and Bamburg, 1979; 1982) or ii) modifies mechanisms involved in the overall *in vivo* control of the assembly of the cytoskeleton

(Coss *et al.,* 1982). The exact mechanism of heat induced alterations in cytoskeletal organisation are yet to be elucidated. It was suggested that the cytoskeleton may provide a structural continuity between the plasma membrane and the nucleus, and as hyperthermia induced changes in at least one skeletal element in all cell types studied so far, hyperthermia-induced disruption of the cytoskeleton may play a role in the heat-induced increase in nuclear protein content (Roti Roti and Laszlo, 1988).

Increasing attention has been focused on membranes as being a site for hyperthermic damage to cells (Hahn, 1982; Bowler, 1987; Konings, 1988; Yatvin and Cramp, 1993; Bowler and Manning, 1994). The question arises why membranes and membrane function should be susceptible to perturbation by heat? Both the lipid and protein moieties of membranes should be considered from this respect. The current accepted model of biological membrane is a modification of the fluid mosaic model proposed by Singer & Nicholson (1972), with lipid molecules forming a fluid bilayer matrix in which proteins are dispersed. Lee and Chapman (1987) and Cossins and Raynard (1987) both emphasise that a change in temperature will pertubate both the lipid and protein moieties that make up membranes.

Lee and Chapman (1987) reported that a change in temperature has two effects on membrane lipids. First, by gradually increasing temperature the molecular motion of the lipids present will increase, the result will be a progressive increase in fluidity. The second effect is to cause a change in phase from gel to liquid-crystalline. Below the transition temperature the fatty acyl chains of the phospholipids are packed in an ordered form, and melting occurs because of the thermally-induced flexing of their acyl chains. Above the transition temperature the bulk of the lipids are liquid-crystalline and this state is considered to be essential for the function of, and lateral mobility of, integral membrane proteins (see Stubbs, 1983).

Poikilothermic animals respond to change in environmental temperature by altering the degree of saturation of their membrane lipids (Hazel and Prosser, 1974; Cossins and Sinesky, 1984), in response to a change in rearing temperature. Acclimation to low temperature leads to incorporation of unsaturated fatty acids into phospholipid fraction in the tissue, whereas at higher environmental temperatures, a great proportion of saturated fatty acids are incorporated, an effect that occurs in a graded response to temperature change (Hazel and Zerba, 1986). This is interpreted as a compensatory adaptive response to the temperature change which preserves membrane lipid order, and has been termed homeoviscous adaptation (Sinesky, 1974). The functional importance of this response is that the properties of plasma membranes are buffered, over the medium term, against the direct effect of temperature change. This implies that membrane protein function is responsive to the lipid environment (Cossins, Beham, Jones and Bowler, 1986). This idea that organisms change the lipid composition of their cell membranes to compensate for direct effects of temperature on membrane physical properties, was an important step in appreciating that temperature has a powerful modulating influence on membrane structure, (Hahn, 1982; Dewey, 1983). Furthermore, there is considerable evidence of changes in the degree of cellular lipid saturation in response to temperature change in diverse organisms e.g. in poikilotherms (Cossins, 1994).

Implicit in the concept of homeoviscous adaptation is that normal cellular function requires cell membranes to possess a specific level of fluidity. Thus, the functioning of membrane proteins might be regulated by, and be dependent on, membrane fluidity, (Cossins and Sinesky, 1984). If lipids are involved in cellular heat killing then clear evidence argues against this being a consequence of a gel to liquid-crystalline phase change of the bulk membrane lipid (Lepock, Cheng, Al-Qysi and Kruuv, 1983),

although such phase transitions have been shown to correlate with hypothermic cell death, impaired growth and cell division at low temperatures, in mammalian cells (Lepock *et al.,* 1983; Kruuv, Glofcheski, Cheng, Al-Qysi Nolan and Lepock *et al.*, 1983), and in microorganisms (McElhany, 1985). However, it can not be excluded that a small critical fraction of membrane lipids are involved in gel to liquid-crystalline transitions at hyperthermic temperatures.

An interesting extension of the fluidity hypothesis suggests that hyperthermic temperatures have a deleterious hyperfluidizing effect on membrane lipids (Overath, Schairer & Stoffel, 1970; Yatvin 1977). Dennis and Yatvin (1981) have produced a good correlation between microviscosity (fluidity) and sensitivity to hyperthermia in an unsaturated fatty acid requiring mutant of *Escherichia coli.* The relationship in eukaryotic cells in much less clear, Lepock, Massicote-Nolan, Rulle and Kruuv (1981); Yatvin, Vorphall, Gould and Lyte (1983).

It is debatable however whether there is a causal relationship between membrane fluidity and susceptibility of cells to hyperthermia for it would be expected that there should be a direct relationship between cell survival and measurement of membrane fluidity in response to the heating (Lepock, 1981; Konings, 1985; 1988). However, Dynlacht and Fox (1992) have recently shown that whilst there is no relationship between cell survival and initial membrane fluidity in a variety of Chinese hamster ovary (CHO) cell lines exposed to heat, there is a positive correlation with the extent to which fluidity is increased by the heating.

A number of workers have found a positive correlation between growth temperature and cell heat resistance, and this adaptation response is marked in fish FHM cells (Schmidt, Laudien and Bowler, 1984). Anderson, Minton, Li and Hahn (1981) have also shown that mammalian cells show this adaptation response to growth

temperature. Gonzalez-Mendez, Minton and Hahn (1982) have also reported that Chinese hamster ovary fibroblasts grown at 32, 37, 39 or 41°C show a progressive increase in thermal resistance to exposure at 43°C. These workers also found that growth at higher temperatures (39 & 41°C) caused a decrease in membrane fluidity that correlated with the increase in thermal resistance. These studies show that animal cells, both mammalian and ectotherm alter their plasma membrane lipid composition in response to a change in growth temperature, and that this is associated with a predictable change in membrane lipid order, and also a change in the thermosensitivity of cells (Bowler and Manning, 1994).

Changes in cell calcium may affect blebbing of the plasma membrane, which is a common cellular response to hyperthermic injury as well as other forms of injury (Trump and Berezesky, 1987). However it is not clear whether calcium is involved in cellular heat damage for significant discrepancies exist between various studies (Vidair and Dewey, 1986; Yi, Chang, Tallen, Bayer and Ball, 1983; Ruifrok, Kahon and Konings, 1985).

Based on the findings reported for bacterial, ectothermal and mammalian cells it seems that membrane lipid composition, cholesterol content or membrane fluidity are not in themselves the primary factors which determine hyperthermic sensitivity.

Several investigators have pointed towards membrane proteins as the critical target for heat. This conclusion is supported from a variety of reports. Lepock *et al.*, (1983) demonstrated irreversible membrane protein unfolding in heated Chinese hamster ovary cells, membrane active agents interacting with membrane proteins and inhibiting the activity of the sarcolemma membrane bound enzymes, Na^+ K^+ ATPase and Ca^{2+} ATPase (Roed and Brodel, 1981). Furthermore, crayfish muscle membrane ATPase was inhibited in the temperature range lethal to the animal (Cossins and

Bowler, 1976). In subsequent studies it was also shown that the thermal inactivation of synaptic membrane Na^+ K^+ ATPase and acetyl-cholinesterase were dependent on organism acclimation (Cossins, Bowler and Prosser, 1981; Bowler, 1987).

Lepock *et al* (1983) have reported from the measurement of intrinsic protein fluorescence of the energy transfer from protein fluorophobe to transparanaric acid demonstrated the existence of an irreversible transition in protein structure above 40°C, both in mitochondrial and in plasma membranes (Lepock *et al.,* 1983). The latter authors hypothesised that the alterations in the structure of the protein above 40°C could cause many of the observed changes in the plasma membranes and may be involved in hyperthermic cell killing.

Alteration in organelles such as lysosomes and mitochondria during heating have been reported by several groups and these organelles have been suggested as possible targets in heat-induced cell death (Hahn, 1982). For example, morphological changes are observed in mitochondria which may be related to the inhibition of respiration in heated cells (Mondovi, Agro, Rotilio, Strom, Moricca and Rossi-Fanelli, 1969; Christiansen and Kvamme, 1969; Dickson and Calderwood, 1979). Structural changes within the mitochondrial membranes of blowfly flight muscle, following exposure to lethal heat, were observed by Davison (1971b), and it was suggested that these morphological changes may be related to the inhibition of respiration in heated cells. Welch and Suhan (1985) also have reported a number of structural changes in rat fibroblasts mitochondria exposed to 42°C for 3 hours; mitochondria were swollen, the cristae were more prominent, and the intracisternal spaces appeared enlarged. Similar alterations in the structure of the mitochondria have been noted in chick myoblasts exposed to various uncouplers of oxidation phosphorylation (Buffa, Guarriera-Bohyleva, Muscatello and Pasquali-Ronchetti, 1970). Chinese hamster ovary (CHO)

cells grown as a monolayer also were found to have swollen mitochondria after exposure at 41.5°C (Coss, Dewey and Bamburg, 1979). These observations suggest that heating may cause disruption of the inner mitochondrial membrane function, destroying the proton motive force, which is implicated in the energy conserving synthesis of ATP (Mitchell, 1979).

The phenomenon of thermotolerance in cells was originally reported by Gerner and Schneider (1975) and Henle and Leeper (1976) and it has been substantiated by many investigators (Landry *et al.*, 1987; McAlister and Finkelstein, 1980; Mivechi and Li, 1985; Subjeck, Sciandra and Johnson, 1982). The various studies in this field of acquired thermotolerance have been reviewed by Hahn and Li (1990) and Hightower (1991). The biochemical mechanisms involved in thermotolerance have been subject to intense investigation.

The present study continuous earlier work of Davison and Bowler (1971) and Bowler and Kashmeery (1981) who reported that *in vivo* heating of adult blowflies caused the impairment of the functional efficiency of flight muscle mitochondria. Davison and Bowler (1971) showed that sarcosomes have an equivalent thermal sensitivity to that of the whole animal. Using both pyruvate and glycerol-3-phosphate as substrates, it was found that sarcosomes, isolated immediately following sub-lethal heat treatment, were dramatically affected as compared with sarcosomes isolated from control untreated animals (Davison and Bowler, 1971; Bowler and Kashmeery, 1981). After lethal heat treatments (LD_{50} and LD_{100}) they reported that respiratory control, phosphorylation and oxidation were impaired .

Since glycerol 3-phosphate is known to be one of the major substrates of flight muscle sarcosomal respiration (Sacktor, 1958; Van den Bergh, 1962), a breakdown of the phosphorylation process coupled with the oxidation of this substrate, could result in

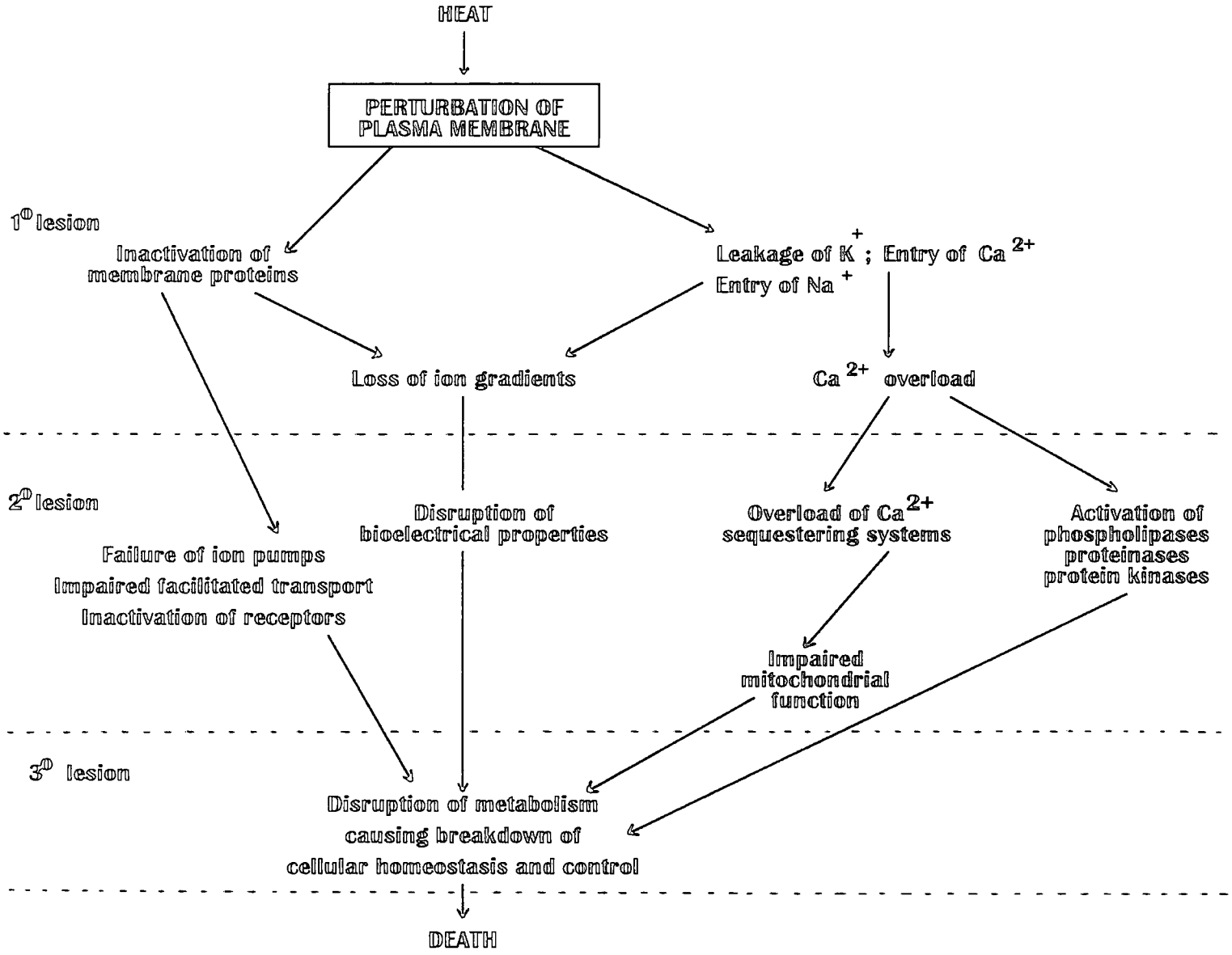
serious effects on the animal. Furthermore, Davison (1971b) demonstrated that there is a correlation between the physiological lesions observed with the sarcosomes isolated from animals exposed to lethal heat treatments and morphology impairment of the sarcosomes, as revealed by electron microscopy. This damage consisted of the disruption of the lamellate cristae and the appearance of a considerable amount of electron dense granules which was attributed to the precipitation of inorganic ions. These results led to the suggestion that sarcosomal sensitivity may be one of the primary causes of heat death in the adult insect. Moreover, these results satisfied one of the criteria for a primary lesion in that a very close correlation existed between the age-specific heat sensitivity of the flies, and the thermal damage caused to the sarcosomes by a particular heat dose. Furthermore they reported that recovery from sub-lethal (LD_0) and the lethal exposure of blowflies required between 2 and 3 days at 24°C. After this recovery period the flies had flight muscle sarcosomes with the same QO_2 values and RCI and ADP:O ratios as did sarcosomes from control unheated flies.

It is significant in the earlier work that in flies that recovered from LD_{50} dose the ability to fly and the restoration of normal mitochondrial function had the same time course (Davison and Bowler, 1971) and the same temperature dependency (Bowler and Kashmeery, 1979). This implies that the mitochondrial impairment observed *in vitro* is also expressed *in vivo*.

The purpose of the present study was to demonstrate that heat shock induces thermotolerance in blowflies, and also that in the thermotolerant state mitochondrial function is protected from the subsequent *in vivo* lethal heat exposure.

In the first instance, studies on recovery from lethal and sublethal heat treatments were carried out. Secondly, biochemical work on the thermal sensitivity of isolated sarcosomes is presented.

Figure 1.1 Scheme to show possible sequence of events during the thermal death of animal cell. (Bowler, 1987).


HEAT
PERTURBATION OF PLASMA MEMBRANE
1° lesion
Inactivation of membrane proteins
Leakage of K+ ; Entry of Ca2+
Entry of Na+
Loss of ion gradients
Ca2+ overload
2° lesion
Disruption of bioelectrical properties
Overload of Ca2+ sequestering systems
Activation of phospholipases proteinases protein kinases
Failure of ion pumps
Impaired facilitated transport
Inactivation of receptors
Impaired mitochondrial function
3° lesion
Disruption of metabolism causing breakdown of cellular homeostasis and control
DEATH

CHAPTER II

GENERAL MATERIALS AND METHODS .

The insects used for the majority of this study were male and female *Calliphora vicina* from a stock maintained in our laboratory for at least thirty years, which was originally obtained from a mass-mated stock at the Pest Infestation Laboratory, Slough. Late in this study this stock was lost and was replaced from a stock obtained from the Department of Zoology at Cambridge University. Studies using this latter stock are specifically identified in Chapter 6.

2.1 Culture of stock flies:

The stock culture used for breeding were kept in cages covered with muslin, approximately 70 cm × 50 cm × 35 cm, with corrugated cardboard placed in the bottom to allow the flies to right themselves. These cages were kept at room temperature (24 ± 1°C) and the stock cultures were replaced at two monthly intervals. The flies were fed sugar and water *ad libitum* and ox liver was provided regularly as a source of protein and to allow oviposition to occur.

2.2 Breeding of experimental and stock cultures.

Liver covered with eggs was removed from the stock cage and placed in a crystallising dish (15 cm diameter) for incubation. The liver was covered with a wet filter paper to provide a high relative humidity (R.H), Davies (1949). The crystallising dish was covered with muslin and transferred to an incubator maintained at 24 ± 0.5°C to ensure larval emergence during the next 12 hours.

Newly hatched larvae were placed on fresh liver in a crystallising dish filled with sawdust and the previous incubation process was repeated. Larvae were fed for 4-5 days on liver, which was replenished as required. Pupation took place in the sawdust 8-9 days after oviposition. After 24 hours the puparia were collected and placed on fresh dry sawdust in a 750 cm^3 conical flask, plugged with cotton wool, and incubated at 24 ± 0.5°C. Emergence occurred between 8 and 10 days later.

2.3 Acclimatisation of experimental flies.

After Emergence male and female adults were placed in cages housed in a constant temperature room maintained at 24 ± 0.5°C. The air circulation was maintained by an air conditioning unit. Continuous illumination was provided by miniature fluorescent lights. RH. was 30 ± 15%. Where groups of 100 or less were used, they were housed in smaller 22 cm × 22 cm × 22 cm cages constructed from commercial biscuit tins with three sides removed. Two sides were covered with polyglaze and a third had a muslin sleeve attached.

2.4 Chemical reagents:

The chemical reagents used in the present study were either AnalaR grade or were of the highest purity which could be obtained commercially. Solutions were made up in glass distilled water.

CHAPTER III

HEAT DEATH POINTS AND THE ESTABLISHMENT OF THERMOTOLERANCE IN THE BLOWFLY CALLIPHORA VICINA .

3.1 INTRODUCTION

Temperature is commonly believed to be an important factor in limiting the distribution of animals. In view of the sensitivity of animals to extreme temperatures and the manifold strategies employed by them to survive exposure, it can hardly be denied that it is important. Yet the evolution of species, communities and ecosystems is the result of a great variety of influences, both biotic and abiotic, which operate in all sorts of subtle ways, (Cossins and Bowler, 1987). Thus reports of a major role of temperature in limiting distribution usually rely on a circumstantial relationship between a climatological factor and species abundance. However, this is not the only issue, as temperature may have its effect not from a direct lethal effect upon the adult or sub-adult form of a species, but indirectly through effects upon the food supply or upon a predator, parasite or competitor. Thus, although many animals live close to their lethal limits, temperature is seldom the primary determinant of their distribution.

Various parameters have been used to establish the temperature limits of organisms, the upper and lower lethal temperatures for species define the temperature limits for survival on exposure to high or low temperatures for a fixed period of time (Bursell, 1964; Fry, 1967; Cloudesly-Thompson, 1970). They are regarded as the physiological limits for the organism beyond which recovery is impossible.

Lethal temperature studies are time consuming and require large number of animals. It is also often difficult to determine whether the animal is actually dead or simply comatose. Consequently there has been a tendency to replace lethal temperature studies with the estimation of critical thermal maxima (CTMax) and minima (CTMin). The critical thermal maximum of a species is a measure of its thermal tolerance and can be used to determine its ability to acclimate to changes in temperature and photoperiod. The initial definition of CTM by Cowles & Bogert (1944) was modified

by Lowe and Vance (1955), who stated that the CTM of a population is the arithmetic mean of the collective thermal points at which locomotory activity becomes disorganised and the animal loses its ability to escape from conditions that, if continued, will quickly lead to death. This has been redefined by Hutchison (1961) and revaluated by Paladino *et al* (1980). The CTM_{ax} should be considered of ecological value since all animals can fully recover from an exposure to CTM, if removed to a lower temperature immediately after exposure. The lethal temperatures are therefore distinct from the CTM because they form the point at which the animal is physiologically dead and cannot recover if placed at a moderate environment temperature. (see Fry, 1967). Whilst CTM gives a measure of thermal resistance, which may have more relevance to the experience of animals in their normal environment than does LD_{50}, it has the disadvantage that the change in temperature is so slow that the resistance of an animal may actually change during the experiment.

The commonest method of defining the lethal conditions of temperature and time for a group of animals is to determine the combination of temperature and exposure time which kill a given percentage, say 50% of the sample, this can be done in two ways; a) by exposing the animals to a single lethal temperature and monitoring mortality with increasing periods of time, or b) to monitor the mortality of animals exposed to different lethal temperatures for a given exposure period. In each case the percentage mortality is plotted as function of time or temperature respectively, and the time or temperature for 50% (the median lethal dose or LD_{50}) is estimated graphically.

In the case of time mortality, sigmoidal graphs are usually obtained which illustrate the statistical nature of thermal mortality, i.e. most of the animals die over a fairly restricted range of lethal conditions, but some succumb quickly whilst others are less susceptible.

A second problem with the LD_{50} type of experiment is difficulty in the establishment of a suitable criterion of death. Clearly, the most unequivocal procedure is to expose samples of animals to a lethal temperature for a specific period and then return them to their holding temperature so that the proportion killed can be estimated by counting the number that fail to recover. This has the disadvantage of requiring large numbers of animals as each temperature-time combination requires a separate sample of animals. However, it is unavoidable when the lethal conditions of eggs or other immobile stages of a life cycle are being determined.

Alternatively, it is possible to use a symptom of thermal death, such as the loss of righting response or the cessation of respiratory movements, and to determine the time taken for that point to be reached.

The influence of temperature on the survival of insects has been investigated by many workers, and the literature contains a great deal of data concerning the ability of insects to withstand exceptionally high temperatures (e.g. Uvarov, 1931; Wigglesworth, 1965; Cloudesly-Thompson, 1970).

It is widely reported that the temperature tolerance limits of insects are influenced by a number of factors, such as age (Baldwin, 1954; Hollingsworth and Bowler, 1966; Davison, 1969), previous thermal history (Bowler and Hollingsworth, 1965), sex (Anderson & Horsfall, 1965) as well as environmental conditions such as humidity (Aelian and Ecksrand, 1975) and stadium (Davison, 1969). These factors must be taken into consideration in designing techniques for testing temperature tolerance of a species.

Adaptive responses that permit normal activity over an environmental range can occur only within limits imposed by the genotype. Within these limits, animals are capable of acclimation or acclimatisation, which can also complicate studies of thermal

resistance. The ability to acclimatise has been demonstrated in a variety of insect species, however compared to other poikilotherms they acclimatise relatively poorly (Bursell, 1964). Walche (1948) showed that chironomids collected from streams below 15°C had less resistance to heat than individuals taken from still water at 20°C. Fraenkel and Hopf (1940) showed that flesh fly larvae reared at 18°C higher than the controls had, for the same exposure period, lethal temperatures higher by one degree than larvae reared at the lower temperature. Baldwin (1954) showed that *Dahlbominus fuscipennis* when reared at 29°C was more resistant to temperatures between 40 and 46°C than when reared at 17 or 23°C. Annila and Perttunen (1964) showed that the resistance of *Blastophagus piniperda*, to high temperatures also depended on acclimation.

In a comprehensive study on the thermal tolerance of two species of dragon fly nymphs (*Libellula auripennis* and *Macromia illinoiensin*) Martin, Garten and Gentry (1976) demonstrated a variety of factors affected the measurement. They showed that acclimation temperature accounted for about one-third of the variation found. Other factors such as body size, time of the day and rate of heating had significant affect on thermal tolerance.

Mutchmore and Anderson (1971) showed that the rate of temperature acclimation could also relate to life style in insect species. They described that *Tribolium confusum*, which lives in a stable environment, attained acclimation slowly, whereas *Musca domestica*, which inhabits variable thermal habitats, attained acclimation rapidly.

Maynard Smith (1957) proposed two types of resistance adaptation in adult *Drosophila subobscura*. First, developmental acclimatisation, which is dependent upon the temperature at which the pre-adult stages have been maintained. It is carried over into the adult and seems to be developmentally fixed, for it is long lasting. Secondly,

physiological acclimatisation, is attained in the adult stage as a result of being placed in a new temperature regime. It is transitory lasting only as long as the new thermal conditions and seems to be reversible. Both forms of acclimation can contribute to heat resistance of the adult stage.

Davison (1970) has shown in *Calliphora erythrocephala* that acclimation was partially obscured by the changes in temperature tolerance which occurred in the young adult. He observed that the LD_{50} of adult *Calliphora erythrocephala* declined after eclosion at a rate which was dependent upon the temperature at which the adult is maintained. The temperature dependence of this loss in resistance indicated that it is metabolically dependent. Thus, when flies were transferred on emergence at 24° to 5° C, no decline in LD_{50} had occurred after 10 days, but when transferred from 24° to 29°C the LD_{50} has declined to give a minimum value after 2 days. However, transfer to a higher temperature still (32-34°C) allowed acclimation to overcome the developmental decline in LD_{50} (Davison, 1970). Bowler (1981) explained this as an example of paradoxical resistance acclimation.

A number of intrinsic and extrinsic factors have been shown to affect the thermal tolerance of insects. Age and stadium are such factors. In a very early study Baldwin (1954) presented evidence which indicated that the heat death point of *Dahlbominus fuscipennis* was related to the age of the emerged adult. Davison (1969) found the egg stage of *Calliphora erythrocephala* is the most sensitive to temperature and the puparium stage most resistant. In the 1-day adult heat death is as great as in 1-day puparium. As has been discussed above these changes may be part of more general physiological changes in resistance to both physical and chemical stresses. Furthermore, Davison and Bowler (1970) have interpreted this decline in tolerance as a loss of pupal heat resistance. It is not peculiar to the blowfly, for it has also been

described in *Drosophila.* Bowler and Hollingsworth (1965), during work on the effect of inbreeding in *Drosophila subobscura*, observed a decline in resistance to 34°C after 7 days of age in the inbred B & K strains. Later work by Hollingsworth and Bowler (1966), on these inbred insects confirmed that a rapid decline in resistance to 34°C occurred in the young adult clearly as a result of developmental changes rather than from senescence. Bowler (1967) has also shown similar but more dramatic changes in temperature tolerance in *Tenebrio molitor*, this suggested that these changes may well be a widespread phenomenon in endopterygote insects. Evidence also shows that heat tolerance in insects decreased with increasing age. For example, Lamb & McDonald (1973) showed that heat tolerance changed with age in normal and irradiated *Drosophila melanogaster* kept at 35°C in dry air. More recently, Niedzwiecki, Kongpachith and Fleming (1991) studied the effect of cellular ageing on adult mortality in *Drosophila melanogaster* under thermal stress. Their results showed that flies exposed to 37°C for various time intervals had reduced survival rate with age, which suggested that old flies are more sensitive to thermal stress than young ones.

Humidity also affects tolerance to high temperatures in insects. A number of examples quoted in Edney (1957), have demonstrated higher temperature tolerance in dry air than in saturated air over short exposure periods, however, with longer exposure periods the effect is reversed, as the harmful effects of desiccation begin to outweigh the beneficial effects of cooling from evaporation of water. Small arthropods tolerate high external temperatures better in moist environments, this is because the volume of body water available for evaporative cooling is to too small to be an effective protection against high temperature, By contrast large insects can use evaporation of water for cooling, and therefore withstand higher air temperature exposure in dry air. It is estimated that a large insect can afford to loose up to 20 -40%

of its body weight before desiccation became a crucial factor. *Periplaneta*, for instance, dies at 38°C at high humidities, but can survive up to 48°C if the air is dry. *Blatta* also can survive for 24 h at 37-39°C if the air is moist, but die as a result of similar exposure in dry air (Appel, Reierson and Rust, 1983). Thus, exposure to high temperatures can be associated with the risk of desiccation, and evidence suggests that death occurs as a result of cumulative loss of water (Maynard Smith, 1956). Furthermore, the effectiveness of evaporative cooling decreases as humidity increases (Prange and Pinshow, 1991; Toolson, 1987; Toms, 1986; Seymour, 1974). For this reason all heat exposure in our experiments were made with humidity controlled at about 50% R.H.

Thermotolerance can be distinguished from acclimation mainly on temporal grounds. The former is triggered by short exposure to high lethal or sub-lethal temperatures and usually requires time at a viable temperature to develop the resultant transient increased heat tolerance (Schlesinger, Tissieres and Ashburner, 1982; Subject and Shyy, 1986; Rutledge *et al.*, 1987). Acclimation, on the other hand develops following a rise in environmental temperature within viable limits, over a relatively long time course. In insects it may take only a few hours at the new temperature (Colhoun, 1960) but more usually it requires days or weeks to develop (Bowler and Hollingsworth, 1965).

Early studies on thermotolerance in insects, as a result of exposure to supra-optimal temperatures, has been clearly demonstrated in flies (Ritossa, 1962; Milkman, 1962; Mitchell *et al.*, 1979). Ritossa (1962) described that temperature and DNP shock in *Drosophila* caused activation of some genes, whereas others were less active in the 2L and 15 regions of salivary gland giant chromosomes of *Drosophila bucksii*. These changes were visible as the appearance or disappearance of so-called puffs. Later, the

protection products of these activated genes were purified from *Drosophila* by Tissieres *et al* (1974), and called heat shock proteins (HSPs).The heat shock response has been characterised in other (Diptera) *Chironomus tentans* (Vicent and Tanguay, 1979); *Sarcophaga bullata* (Bultman 1986); *Aedes albopictus* (Carvallo and Rebello, 1987); in *Locusta migratoria* (Orthoptera) (Whyard, Wyatt & Walker, 1986); in *Periplaneta americana* (Dicyoptera) (Ruder, Ovsenek, Heikkila and Downer (1989); and in a few lepidopteran species e.g., *Calpodes ethlius*, Dean and Atkison, 1983).

The induction of thermotolerance has also been observed in the pharate adults of the flesh fly *Sarcophaga crassipalpis* after the brief exposures to supraoptimal temperatures (35-45°C) (Yokum and Denlinger, 1992). Ninety minute exposure to 45° C was normally lethal to flies reared at 25°C, but a brief pretreatment at high temperature generated protection from subsequent heat shock injury. In *Sarcophaghaga crassipalpis* the induction of thermotolerance was dependent upon both temperature and duration of the pretreatment, it was induced by a 2 h exposure to 40°C and decayed slowly over 72 h at 25°C. Additional evidence of this phenomenon has been described in other insect species e.g. in the Mediterranean fruit fly *Ceratatis capitata* (Stephanou, Alahiotis, Mamarus and Christodoulou, 1983). In this work, exposure of larvae at 37-43°C for 40 min. did not affect survival rate, but heat shock at 45°C for 40 min. reduced it, and at 47°C all larvae were killed. However, if the animals were preheated at a mild temperature 35°C for 30 or 60 min and then subjected to a nearly lethal temperature (45°C) a dramatic enhancement of survival was observed. Parallel to this effect, when *Ceratitis capitata* received pretreatment of a mild temperature 35°C for 40 min a great enhancement of HSPs production was observed. The description and characterisation of these protein patterns in *Ceratitis capitata* has been studied in detail by Stephanou *et al.* (1983).

The phenomenon of thermotolerance was related, by Mitchell, Moller, Peterson and Lipp-Sarmento (1979) with the induction of HSPs during hyperthermic treatment. They found that preheating at a non-lethal temperature (30 min at 35°C) improved both the ability of *Drosophila melanogaster* larvae, adults and cell lines to survive a normally lethal heat stress and the prevention of the induction of developmental defects (phenocopies). This dramatic effect on survival and phenocopy prevention were correlated with a much more rapid recovery of protein synthesis in the animals that had received the 35°C preheating (Mitchell *et al.*, 1979).

The present study continues earlier work of Davison and Bowler (1971) and Bowler and Kashmeery (1981), the main objectives of the work described in this chapter were:

1. To determine the LD_{50} point for the stock of adult *Calliphora vicina* used.

2. To determine if thermotolerance can be induced by pretreatment at sub-lethal high temperatures.

3. To follow the time-course of any induced thermotolerance.

3.2. MATERIALS AND METHODS.

3.2.1 Rearing of experimental animal:

Throughout this part of the study animals were reared as described in chapter 2, at a constant temperature of 24 ± 0.5°C in a R.H. of 50%.

3.2.2 Determination of the heat death points:

Groups of 100, 10-day old flies from both stocks were given a standard 40 min exposure to a series of temperatures, at 0.5°C intervals, within the range of 36.5 to 40° C, for Durham or 41°C for Cambridge stocks. Control mortalities were determined using flies held under the same conditions, but without heat treatment. Heat treated flies were returned to culture conditions to allow their recovery.

Evidence from preliminary experiments showed that deaths in adults caused by heat treatment occurred on the 2 days following heat exposure. Survivors were then fully active and behaving normally. Animals showing no movement and no reaction to stimulation, two days after treatment, were counted dead, and the heat death point was determined as the temperature dose that kills 50% of the experimental flies, see section 3.2.5.

3.2.3 Method of exposure of flies to high temperatures .

The animals were placed in a series of 7.5 cm x 2.6 cm glass vials, which were completely immersed in a water bath at the required temperature. Racks were constructed to hold 50 vials. Each vial fitted to a rubber bung, connected by glass and rubber tubing as shown in Figure 3.1. Air was brought to a R.H . of 47.5 - 50.5 per cent by bubbling through a saturated solution of Mg (NO_3)$_2$ (Winston and Bates,

1960) before circulation through the vials at 600 cm^3 per min. These exposure conditions were chosen to maintain consistency with earlier work (Davison & Bowler, 1971 and Bowler and Kashmeery, 1981).

The temperature of the water bath was maintained at ±0.05°C using an immersion heater (Templete Junior TE-8J). The water bath was continuously stirred. In the temperature range used, 15 min. was required for equilibration of the air and water bath temperature (Davison, 1970). This 15 min period was included in the 40 min. exposure period. During the remainder of the exposure the air temperature was maintained at the temperature of the water bath ± 0.05°C. After exposure to experimental temperatures the flies were transferred into small cages 22 cm x 22 cm and placed in the same room as the same stock of flies (i.e.) at 24 ± 0.1°C 50% R.H., sugar and water were provided. Typical temperature / mortality curves are shown in Figures 3. 2 and 3.3 and Table 3.1.

3.2.4 Establishment of thermotolerance:

These experiments were designed to determine the extent and time-course of the development of thermotolerance. Male and female blowflies of both Durham and Cambridge stocks were used in these experiments and were reared and maintained at 24 ± 0.5°C using culture methods previously described in Chapter 2.

One hundred and twenty 10-day old flies were used in this study. These were exposed for 40 min. to 36 ± 0.1°C (sublethal or shock temperature) for Durham stock and 37°C for Cambridge stock, again following methods previously described in section 3.2.3 of this chapter. Following the completion of the heat-shock flies were transferred to 24°C for a period of time varying from 1 hour to 6 hours. After the specified recovery period the flies were again placed in vials and given the LD_{50} dose of

38.12°C (Durham stock), or 39.54°C for 40 min (Cambridge stock). A second batch of 120 control flies were heated simultaneously with the heat-shocked flies, after which they were returned to 24 ± 0.1°C and allowed to recover. The recovery period allowed was 2 days at 24°C and the number of dead flies were counted. This experiment was repeated 10 times and the data were combined. The mean values were scored and plotted as a function of recovery at each temperature, Figure 3.4 and Table 3.2.

3. 2.5 Method of analysis of results:

Mortality after heat treatment was recorded, as described previously. The heat treatments were given over a range of temperatures and the relationship of mortality to temperature was obtained. In both stocks of flies the graphs gave a sigmoidal curve (Figure 3.2). The fact that, the temperature mortality relationship is similar to a typical dosage effect observed with toxicological and pharmacological data, this allowed the following simple method of conversion of the sigmoid curve to a straight line. The mortalities of unheated control flies was negligible, but was deducted from the mortality recorded after heat treatment. Where M is the mortality after a heat treatment of 100 animals, C is the mortality observed in the untreated control group of 100 animals, and H is the mortality caused by the heat treatment , then

$$H = M - C ;$$

H expressed as percentage H'

$$H' = \frac{Hx100}{100 - C}$$

Mortalities below 1 or above 99 per cent were not considered, so that the slope of the sigmoidal curve could be converted into a straight line using probit analysis (Finney, 1952), originally developed in toxicity testing, and has come to be widely applied in the determination of lethal temperatures. The statistical treatment of these data was carried out as described in Appendix 1. This analysis permitted the estimation of the thermal dose giving 50% mortality (probit 5) and also the determination of 95% confidence limits for the 50% mortality dosage.

3.3 RESULTS:

The Durham stock of flies was lost late in the programme and replaced with different stock obtained from Cambridge. For this reason it was necessary to establish the LD_{50} of both stocks of flies.

The untransformed data of the heat death of adult blowflies from both stocks are shown in Table 3.1 and Figure 3.2. As can be seen from Figure 3.2 the heat dose survival curves were sigmoidal, and that for the Cambridge stock was displaced to a higher temperature range than the curve for Durham stock. These data are shown in Figure 3.3 in the form of probits. The median heat dose can be obtained by construction from probit 5, and was also determined to be 38.16 ± 0.47°C (Durham) and 39.47 ± 0.18°C (Cambridge) for the two stocks of flies, using the method described in Appendix 1. The LD_{50} obtained by construction and also by probit analysis clearly indicate that the Cambridge stock of blow flies is more resistant to high lethal temperature than the original stock of blow flies. But, in general, both stocks of flies show that the survival times decline significantly with increasing temperature.

Figure 3.4 compares the mortality of control flies with those that were subjected to a heat shock prior to testing, for both the original and Cambridge stocks of flies. The test heat dose applied was the appropriate LD_{50} and in both stocks the mortality of control flies did not significantly differ from the expected 50%. However, in both stocks, pretreatment with a non-lethal heat shock led to a time dependent increase in tolerance to exposure to the LD_{50} treatment. The time course of the development and decay of thermotolerance is very similar for the two stocks of flies.

Significant thermotolerance has already developed 1 h after the heat shock, but the full development of this protection required a period of 3 to 4 h at 24°C between the two treatments, when a 30% increase in survival over the equivalent control group was observed. There was then a progressive decay of the developed tolerance which disappeared 6 hours after the heat shock. The experiment has been repeated with the second stock of flies under the same experimental conditions. The initial heat shock was 37°C and maximal acquired thermotolerance is also seen by 3 to 4 h, showing no difference in behaviour from the original stock of flies, while longer intervening periods e.g. 6 also resulted in a gradual decay of the induced thermotolerance.

Figure 3.1 Heating apparatus. Diagram showing the arrangement of the vials on the heating rack.

a, rubber stopper; b, glass vial;

c, rubber tubing; d, glass tubing.

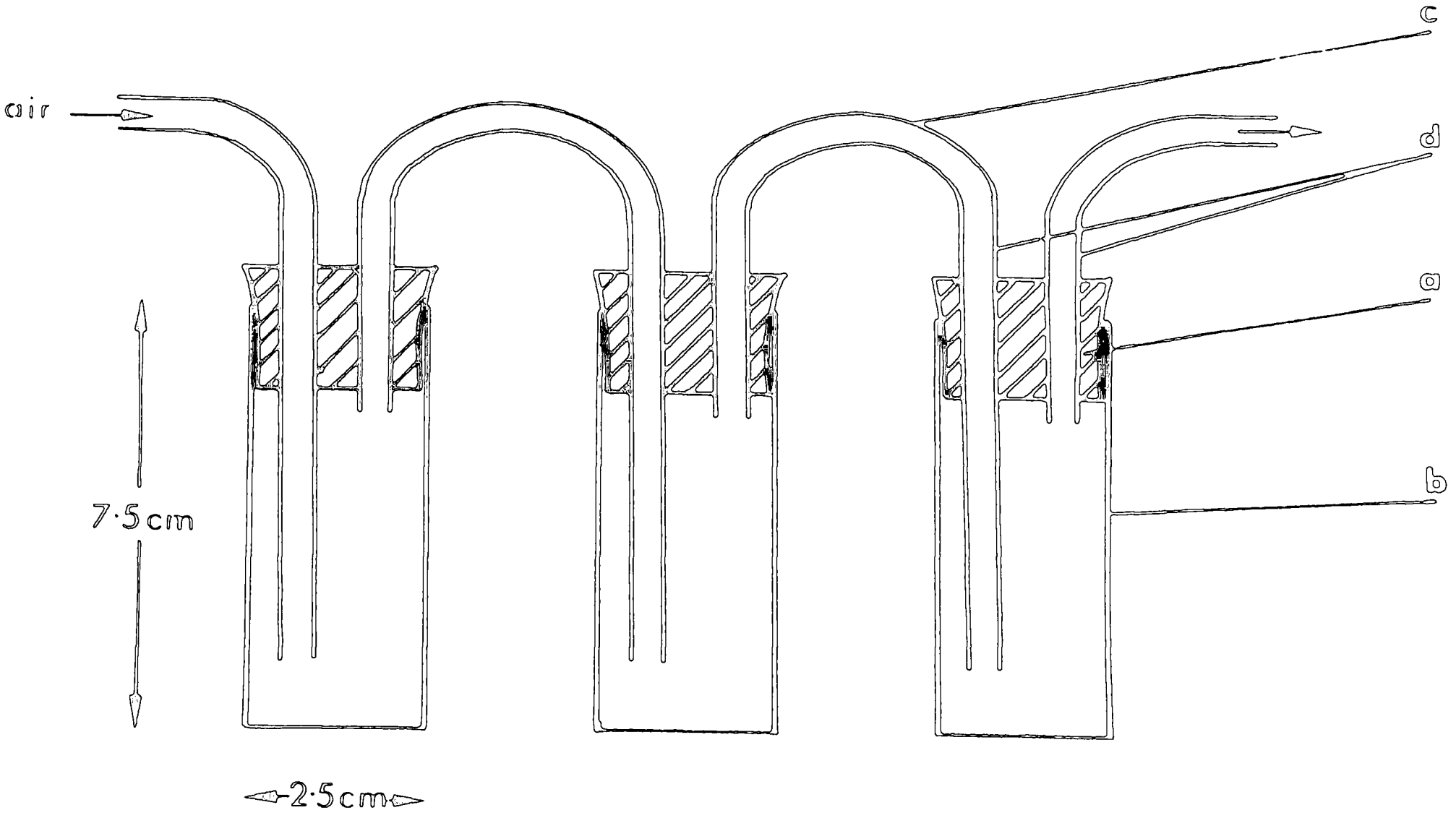

air
c
d
a
b
7·5 cm
2·5 cm

Table 3.1. Heat death points for 10-day-old adult *Calliphora vicina*, using two different stock cultures.

Percentage mortality over the temperature range 36.5° to 41°C for 40 min exposure. Survival determined after 2 days recovery at 24°C. Means ± S.E.M. (N= 10).

Table 3.1. Heat death point for 10-day-old adult Calliphora vicina, using two different stock cultures.

Temperature (°C)	Percentage mortality	
	Original stock	Cambridge stock
42.0	-	96.0 ± 0.42
41.5	-	84.0 ± 0.35
41.0	94.0 ± 0.51	72.16 ± 0.33
40.5	92.0 ± 0.44	58.42 ± 0.21
40.0	85.0 ± 0.40	50.83 ± 0.18
39.5	70.0 ± 0.43	40.16 ± 0.19
39.0	59.0 ± 0.28	35.82 ± 0.22
38.5	53.0 ± 0.22	30.60 ± 0.16
38.0	45.0 ± 0.18	22.50 ± 0.14
37.5	34.0 ± 0.26	20.00 ± 0.26
37.0	31.0 ± 0.16	17.90 ± 0.22
36.5	29.0 ± 0.15	14.83 ± 0.34

Figure 3.2 Dose mortality curve for the two stocks of 10-day-old adults of *Calliphora vicina* as a function of high temperature exposure. The number of survivors was determined after a 2 day recovery period at 24°C. Data from Table 3.1.

.

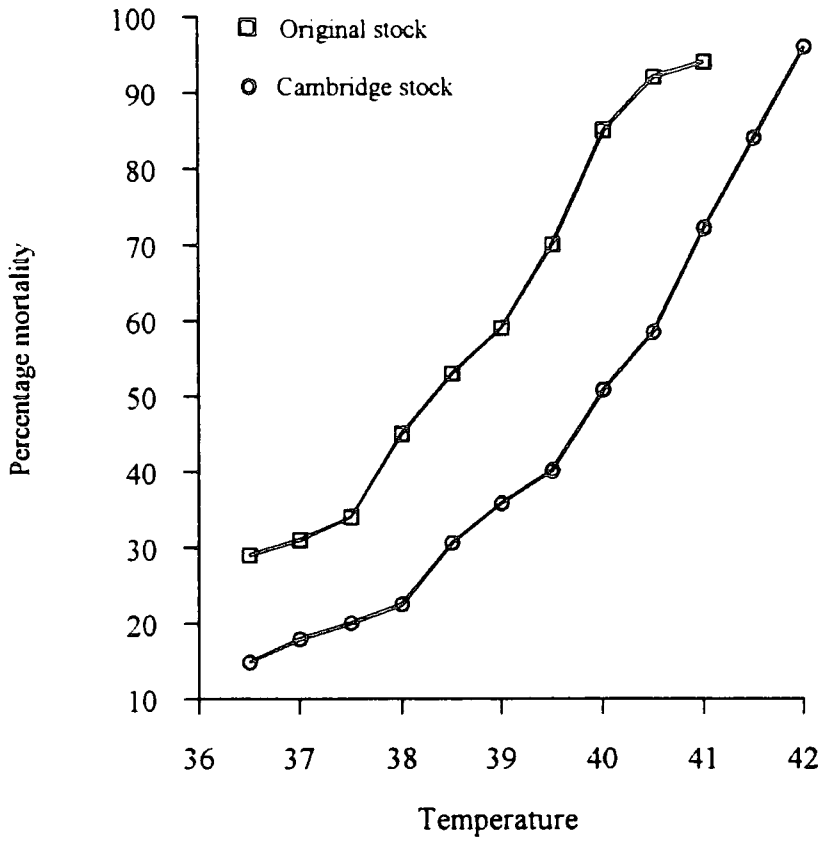

Original stock
Cambridge stock
Percentage mortality
100
90
80
70
60
50
40
30
20
10
36
37
38
39
40
41
42
Temperature

Figure 3.3. Probit analysis of heat death data for the two stocks of adult *Calliphora vicina.*

The percentage mortality data presented in Table 3.1 were converted into probits and lines of best fit were calculated according to the method shown in Appendix I.

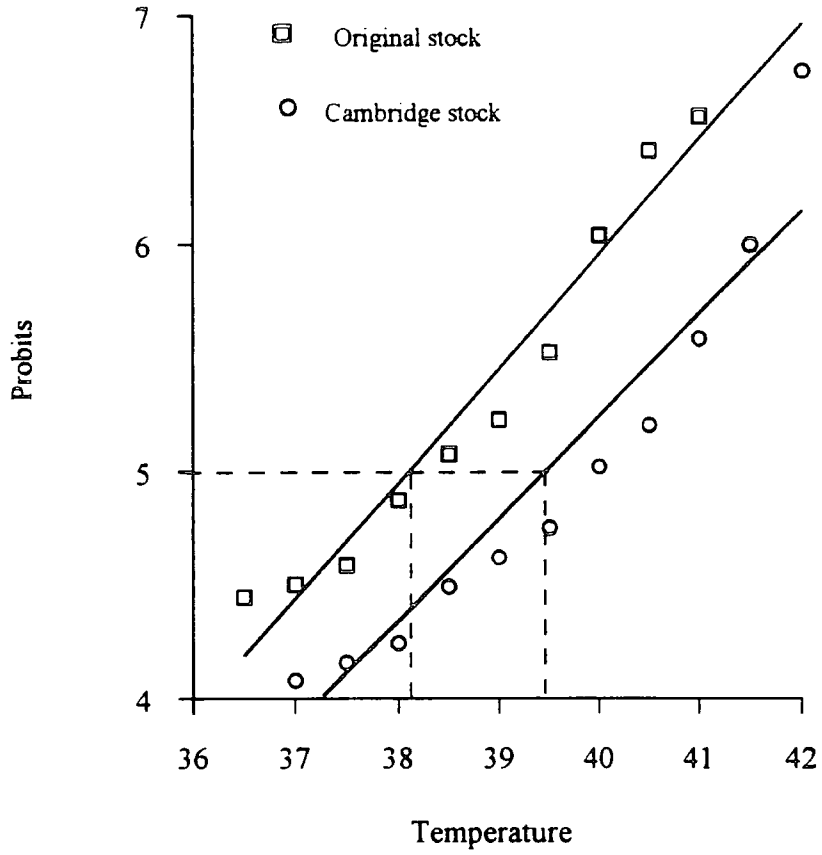

Original stock
Cambridge stock
Probits
7
6
5
4
36
37
38
39
40
41
42
Temperature

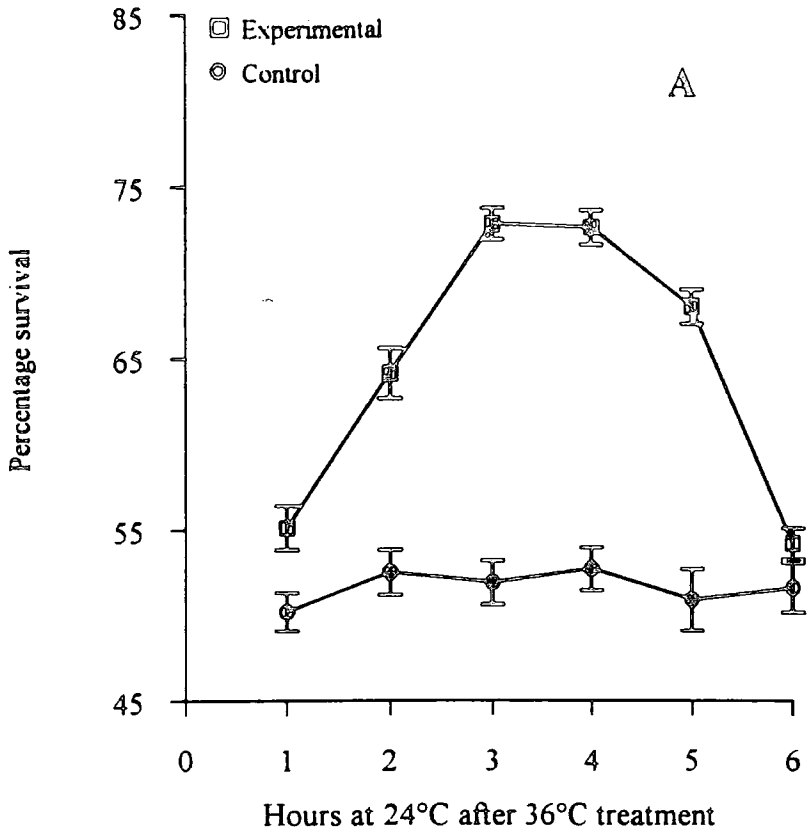
Table 3.2. The time course for the development of thermotolerance. 10-day-old adult blowflies were subjected to a 40 minute exposure at 36°C (original stock) or 37°C (Cambridge stock). Blowflies were then returned to 24°C for 1 to 6 h, to allow the development of thermotolerance, before being subjected to an LD_{50} heat dose (38°C for 40 min, original stock or 39.54°C for Cambridge stock). Data are shown as mean percentage mortality ± S.E.M. N = 10. Control flies were not subjected to heat shock but were exposed to the LD_{50} dose simultaneously with experimental blowflies.

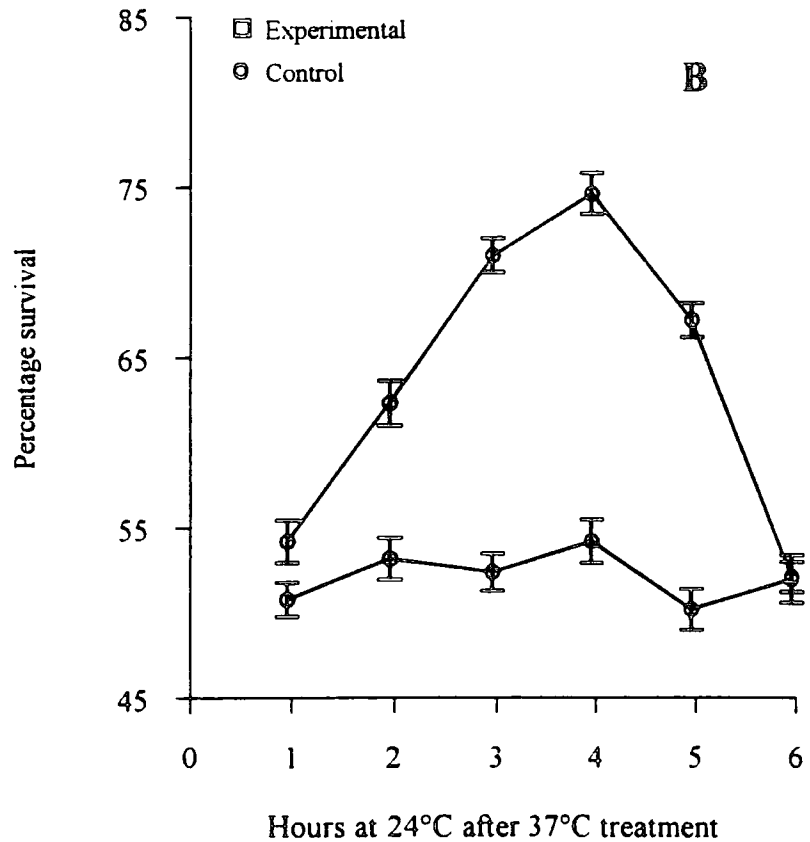
Table 3.2. Time course for the development of thermotolerance. 10-day adult blowflies were subjected to a 40 min exposure at 36°C (original stock) or 37°C (Cambridge stock).

Time at 24 °C (h)	Percentage survival			
	Original stock		Cambridge stock	
	Experimental	Control	Experimental	Control
1	55.1 ± 1.30	50.2 ± 1.10	54.20 ± 1.26	50.8 ± 1.00
2	64.1 ± 1.46	52.5 ± 1.30	62.32 ± 1.30	53.2 ± 1.21
3	72.8 ± 0.90	51.9 ± 1.30	70.96 ± 1.00	52.4 ± 1.10
4	72.6 ± 1.00	52.7 ± 1.24	74.60 ± 1.20	54.2 ±1.30
5	68.0 ± 0.98	50.9 ± 1.80	67.20 ±1.01	50.2 ± 1.22
6	54.2 ± 0.91	51.6 ± 1.45	52.12 ± 0.89	52.0 ± 1.41

Figure 3.4. A: Time course of the development of thermotolerance in 10 day-old adult blowflies, following pretreatment for 40 min at 36°C (Durham) as determined from changes in the LD_{50} of 10-day-old blow flies. The pretreated flies were taken at 1, 2, 3, 4, 5 and 6 h and their LD_{50} determined coincidentally with that of a batch of control flies that had not been pre-treated. Data from Table 3.2. Values are mean 1 ± S.E.M. (N=10).

B: Time course of the development of thermotolerance in 10 day-old adult blowflies following pretreatment for 40 min at 37°C (Cambridge stock) as determined from changes in the LD_{50} of 10 day-old blowflies. the flies were taken at 1, 2, 3, 4, 5 and 6 h and their LD_{50} determined coincidentally with that of a batch of control flies that had not been pretreated. Data from Table 3.2. Values are mean 1 ± S.E.M (N = 10).


Experimental
Control
A
Percentage survival
85
75
65
55
45
0
1
2
3
4
5
6
Hours at 24°C after 36°C treatment


Experimental
Control
B
Percentage survival
85
75
65
55
45
0
1
2
3
4
5
6
Hours at 24°C after 37°C treatment

3.4 DISCUSSION.

The data obtained for LD_{50} for the original stock of 10 day-old blowflies *Calliphora vicina* developed and maintained at 24°C was 38.16 ± 0.47°C and is significantly lower than that determined for a similar stock of blowflies using the same protocol reported by Davison (1969) and by Bowler and Kashmeery (1981). The cause of this difference is not clear, but it may result from an inadvertent selection of less resistance stock over the intervening period, whereas, the results obtained from the Cambridge stock of flies which was 39.47 ± 0.47°C agreed with those of Davison (1969) and Bowler and Kashmeery (1981) in the earlier studies.

Davison (1969) observed that during the heat treatment of *Calliphora erythrocephala* only newly emerged adults showed a significant water loss. One-day-emerged adults lost 5 per cent more water at 43°C than 30-day-old adults. The additional water loss in the young adult may be due to relatively poor waterproofing of the cuticle or poor spiracular control. The fact that the water loss in young adults may represent a more efficient method of evaporative cooling has been discounted. Edney and Barrass (1962) reported that the spiracles of teneral *Glossina morsitans* open above 40°C, and at 45°C they were able to cool their bodies by 1.66°C, when the air was completely dry. In moist air they observed no difference between body and ambient temperature. However, larva and puparia of *Calliphora erythrocephala* posses a higher heat death point than 30-day-old adults and show no water loss (Davison, 1969). It is therefore suggested that the higher heat death point in young emerged adults is due to similar factors to those involved in the heat death point of larvae and puparia. The additional water loss in 1-day-emerged adults is of secondary importance. Water loss during LD_{50} treatment was not determined in this present study, but it is unlikely that heat death reported in this study was due to changes in water content, as

this parameter has been reported to be unaffected by heat treatment in this species (Davison, 1969). The results presented here demonstrate that thermotolerance can be induced in blowflies, as heat damage in *Calliphora vicina* caused by an LD_{50} exposure, was clearly reduced by previous exposure to a sublethal temperature. However, such protection was found to be transitory and time dependent; similar transient thermotolerance due to exposure to supraoptimal temperature has been observed in other insects (Milkman, 1962; Mitchell, *et al.,* 1979; Tissieres *et al.*, 1974; Chen, Richard, Lee and Denlinger, 1991; Carretero, Carmona and Dietz, 1991). The kinetics of the induction of thermotolerance in blowflies indicated that this protection started 1 h after the conditioning exposure, reached a maximum between 3 -4 h and had decayed by 6 h. The description of the heat shock in the insect under investigation has revealed similarities and differences in comparison to that of Drosophila and other taxa in terms of the optimum response, the time course of thermotolerance, and its decay. A similar response is well documented, especially for *Drosophila melanogaster* (Mitchell *et al,* 1987) and *Chironomus thumni* (Carretero *et al.,* 1991).

Tissieres *et al.,* (1974) showed heat shock caused a cessation of the normal protein synthesis. This was followed by the synthesis of specific new proteins which correlate with the changes in the puffing pattern of the chromosomes. The development of puffs on chromosomes is known to be associated with gene transcription, and these workers concluded that heat shock extensively altered gene transcription and consequently gene expression. It is probably for these reasons that the period at which the thermal shock is applied determines the type of phenocopy.

The development of thermotolerance has also been demonstrated in *Sarcophaga crassipalpis* by Yokum and Denlinger (1992) Their studies demonstrated that the induction of thermotolerance was dependent on both the temperature and duration of

the heat shock. The period over which thermotolerance lasted in *S. crassipalpis* was 72 h, considerably longer than the 5 h period found for blowflies in the present study, which is similar to that reported for *Sarcophaga crassipalpis* by Chen *et al.,* (1991). The extent and duration of the thermotolerance developed may be dependent not only on the conditions of the heat shock, but also on the severity of the lethal temperature used.

The present study also demonstrated similarities to *Ceratitis capitata* (Stephanou *et al.,* 1983) where pretreatment at a mild temperature followed by heat shock resulted in an enhancement of survival. Some differences, however, can be seen, in terms of the optimum pre-treatment temperature, that temperature is 37-41°C for *Ceratitis capitata,* while it is 36-38°C for *Calliphora vicina.* This difference may reflect differential preference of the optimum environmental temperature between the two species. A shock of 40°C for 40 min usually kills *Calliphora vicina,* while this stress did not reduce survival of *Ceratitis capitata* (Stephanou *et al.,* 1983).

Whyard *et al.*, (1986) reported similar data to those in this study using *Locusta migratoria.* Adults reared at 27-30°C died after 2 h at 50°C, but they survived this temperature stress if first exposed to 45°C for 0.5 to 4.5 h. The authors suggested that the acquisition of thermotolerance during exposure to elevated sublethal temperatures, must be the result of relatively rapid cellular modification which may be related to the biochemical changes observed during heat shock.

The phenomenon of thermotolerance is not restricted to insects, it has been shown in a wide variety of organisms, for example yeast (McAlister and Finkelstein, 1980); amphibians (Mosley, 1994); crustaceans (McLennan and Miller, 1990); coelenterates (Bosch *et al.*, 1988); sea urchin (Roccheri *et al.,* 1983); reptiles (Maness and Hutchison, 1980); fishes (Otto; 1973); mammalian cells (Henle and Dethlefsen, 1978;

Li and Werb, 1982, Landry *et al.*, 1982). In all cases subjection to a temperature shock prior to that which could kill the animals or (cells) resulted in an enhancement of survival.

Thermotolerance appears to be important for survival under natural stress conditions. A fine example of the latter is the comparison of two species of hydra, *Hydra oligactis* and *Hydra attenuata* (Bosch *et al.*, 1988). The former species is extremely sensitive to temperature and is incapable of acquiring thermotolerance, whereas the latter species is thermoresistant and can become thermotolerant. For example, after pretreatment at 30°C, *H. attenuata* polyps developed thermotolerance and were protected against the deleterious effects of the high temperature. Moreover, after thermal shock *Hydra attenuata* makes a large amount of 60 kD a protein, which is not normally found in *Hydra attenuata.*

Heat hardening may be found routinely in animals which experience large natural diurnal fluctuation in their environmental temperature. Maness and Hutchison (1980) have reported variations throughout the day in hardening in a number of amphibians and fish. The periods for peak hardening were found to correspond with the periods of highest environmental temperatures which supports the adaptive role of hardening.

Rutledge *et al* (1987) demonstrated that the increase in resistance to CTM_{ax} that occurred, in two species of salamander, following heat shock was related to the induction of heat shock proteins. Rutledge *et al* (1987) have expanded the definition of heat hardening suggested by Maness and Hutchison (1980), to include other relatively brief shocks, ones at sub-CTM_{ax} temperatures, but of longer duration (up to one or a very few hours). This Rutledge *et al.* (1987) included in the phenomenon of heat hardening not only the increases in thermal tolerance following the CTM_{aX}, but also

those following one hour sub-CTM_{ax} exposures as well as those following several hours of high field temperature.

Lowe and Heath (1969) have also demonstrated evidence of hardening under field conditions in the desert pupfish, *Cyprinodon mascularis*. Fish frequently entered water at 40-41°C, temperatures that were uncomfortably close to their summer CTM_{ax} (43°C) and well above winter CTM_{ax} (37°C) but, more importantly, higher than the fish can be acclimated to in the laboratory. It is suggested, therefore, that the ability of this fish to enter and spend time at 40°C may be a heat-hardening effect of considerable ecological value.

The data presented for thermotolerance in *Calliphora* are in broad agreement with similar research in other species. However, intra and interspecific variability may exist with regard to optimum treatment, rapid acquisition of thermotolerance and other factors. Despite a great deal of published work dealing with thermotolerance, the molecular basis of this phenomenon remains in debate. The transient development of thermotolerance has been correlated with the production of a set of proteins known as heat shock proteins (HSPs) which were first identified in *Drosophila* (Ritossa, 1962). *Drosophila* at various developmental stages (adult, larvae, tissue or cultured cells) exposed to an acute heat stress have altered patterns of protein synthesis (Ashburner and Bonner, 1979; Tanguay, 1983). The results of Stephanou *et al* (1983) provided excellent correlation between the ability of the fly to survive thermal injury and the rate of the general protein and the heat shock protein synthesis.

Although the biological role of the heat shock proteins has not been elucidated, several lines of evidence show that these proteins are involved in the process for the acquisition of thermal tolerance Mitchell *et al.*, 1979, suggested they protect cells against stress (reviews by Lindquist and Craig, 1986). Nevertheless, the importance of

these proteins (HSPs) in thermotolerance remains controversial and may depend upon the system or organism being studied. Initiation and decay of thermotolerance in *Chironomus thumni* corresponded closely to the induction and degradation of heat shock proteins (Carretero *et al.*, 1991). In contrast, the role of heat shock proteins in thermotolerance of yeast *Saccharomyces cerevisiae* is questionable. Neither culturing *Saccharomyces cerevisiae* with amino acid analogues (which should result in synthesis of non functional proteins) nor with cycloheximide (which blocks protein synthesis) prevented the induction of thermotolerance by exposure to supraoptimal temperature (Hall, 1983). Smith and Yaffe (1991) have also reported that HSPs production is not required for thermotolerance acquisition in yeast.

The prolonged thermotolerance in pharate adults of the flesh fly *Sarcophaga crassipalpis* (which decay slowly over 72 h) also appeared not to be dependent upon the synthesis of HSPs (Yokum and Denlinger, 1992). These workers reported that heat shock protein synthesis stopped, and normal protein synthesis was resumed within the first hour after a 2 h exposure to 40°C. This was clearly shown using pulse-chase experiments because heat shock proteins were synthesised during the exposure period, but were degraded within 24 h after removal from 40°C. Yet thermotolerance persisted beyond 48 h. They then concluded that neither the continuing synthesis nor persistence of heat shock proteins appeared critical in maintaining thermotolerance. The major conclusions drawn from the work described in this chapter as the follows:

1. The heat death point obtained for 10-day-old *Calliphora vicina* stock was 38.16 ± 0.47°C whilst that obtained for the Cambridge stock was 39.47 ± 0.18°C.

2. Development of a transitory thermotolerance in the blowfly *Calliphora vicina* after a brief exposure to elevated temperatures was demonstrated with the same characteristics in the two stocks.

CHAPTER IV

EFFECTS OF IN *VIVO* HEATING OF BLOWFLIES ON THE OXIDATIVE CAPACITY OF FLIGHT MUSCLE MITOCHONDRIA

4.1 INTRODUCTION

Most cells and tissues express their full range of functions only within permissive thermal limits, and the effects of elevated temperatures on mitochondrial respiration and the activities of selected mitochondrial enzymes have been measured for several invertebrates (Smith, 1973; Robb, Hammond and Bieber, 1972; Davison, 1971; Newell and Northcroft, 1967). Such measurements indicated that the functional behaviour of these organelles was drastically impaired by high temperature treatment. Moreover, it was suggested that the thermostability of some mitochondrial enzymes appeared to be correlated with temperature range of the species and that actual enzyme denaturation usually requires unphysiologically high temperatures.

Many enzyme pathways are structurally associated with cell membranes, and the integrity of the bilayer-enzyme relationship is known to be important in conferring orientation upon the constituent enzymes of a pathway and as a consequence, membrane enzymes may be particularly sensitive to thermal perturbation of their function (Bowler, 1987; Yatvin, Dennis, Elegbede and Elson, 1987; Bowler and Manning, 1994). Smith (1973a) has shown that the thermostability of some mitochondrial respiratory enzymes was correlated with the environmental temperature range of the species. This suggests that natural selection is operating on these enzymes at the cellular level .

Mitochondria therefore provide an opportunity to study a more intact system than is available from isolated enzymes, multienzyme complexes, or membrane preparations. The mitochondrion, in particular, represents a highly organised membrane-enzyme system whose functional efficiency is known to be dependent upon the structural integrity and juxtaposition of component enzymes (Lehninger, 1964). Furthermore,

mitochondrial efficiency, as measured by tightness of coupling of oxidation and phosphorylation, can be readily determined. Moreover cellular energy production requires participation of mitochondrial enzymes, and oxidative phosphorylation. Consequently, studies that make use of isolated mitochondria minimise disruption of enzyme-enzyme, enzyme-membrane and enzyme-transport interactions, they are useful in assessing transport capacities, metabolic flux rates, enzyme activities *in situ* and regulation of mitochondrial pathways (Moyas *et al.,* 1990).

Pioneering studies of Lewis and Slater (1954); Sacktor (1954); Van den Berg (1962), demonstrated that mitochondria isolated from insect flight muscle carried out oxidative phosphorylation just as do mitochondria isolated from various mammalian tissues. Oxidative phosphorylation in insects was first demonstrated in mitochondria from *Musca domestica* (Sacktor, 1954), and by Lewis and Slater, (1954) in *Calliphora erythrocephala* mitochondria. That early work has been confirmed by other workers, for example the efficiency of oxidative phosphorylation in respect to the activity of enzymes in insect tissue has been studied in isolated mitochondria of adult insect wax moth *Galleria mellonella* (Wojtczak, *et al.,* 1968), blowflies, *Calliphora erythrocephala* (Tribe, 1967; Davison, 1969; Bowler and Kashmeery, 1981), and *Phormia regina* (Sacktor and Wormser-Shavit, 1966) and cockroach, *Periplaneta americana* and *Blaberus giganteous* (Wojtczak, *et al.,* 1968). These studies all showed that insect mitochondria function similarly to mammalian mitochondria. However, different substrates may be utilised, reflecting the different permeability characteristics of mitochondria from different sources. As a consequence, Chefurka (1966) emphasised some of the parameters that may affect the stability of mitochondria and consequently their respiratory activity, 1) Composition of the isolation and reaction media, 2) Extent of homogenisation, 3) Ageing of mitochondria 4) Other parameters

such as age of insect, concentration of mitochondrial suspension, the order of addition of reactants.

Three different parameters are widely used to characterise the efficiency of oxidation phosphorylation in flight muscle mitochondria. These are the latent ATPase activity, the RCI, and the ADP:O ratios. Mitochondrial damage is often associated with a loss of structural and functional integrity of the inner membrane. This can result in an increase in ATPase activity (later in intact mitochondria), and also an decrease in RCI. This occurs because State IV respiration is increased by damaged mitochondria and State III respiration is usually unaffected, (Van den Berg, 1962).

Glycerol-3-phosphate was used as substrate, because the very high oxidation rates obtained approached the amounts of oxygen consumption during the flight of the blowfly (Sacktor, 1970). For this reason glycerol-3-phosphate has often been suggested as the principal physiological substrate in dipteran flight muscle mitochondria (Sacktor, 1970). On the other hand, other workers (Gregg, Heisler and Remmert, 1962) have maintained that pyruvate also plays a major role in supplying energy for flight, because of the high level of respiratory control obtained. The other Krebs-cycle acids are poorly oxidised by these mitochondria because the mitochondrial membrane is relatively impermeable to them (Van den Bergh and Slater, 1962); this may be due to a lack of carrier molecules (Hansford and Chappell, 1967). It is deduced that both glycerol-3-phosphate and pyruvate must be important in flight muscle metabolism and in consequence a comparison has been made between the two systems to see whether both behave similarly with respect to elevated temperatures.

Mitochondrial function has been reported to be especially sensitive to *in vitro* heating. Morris and King, (1962) first provided evidence that the cytochrome b of the respiratory chain of heart mitochondria was particularly sensitive. This was confirmed

for mouse brain and liver mitochondria by Christiansen and Kvamme (1969). In addition, they also reported differences in thermal sensitivity from different tissues with brain mitochondria being more resistant than those from liver and Ehrlich ascites cells. At the lower temperature used (41°C) they described the first signs of heat injury as being a decline in respiratory control without fall in P : O ratio values, at higher temperatures (45°C), however, they found that the mitochondria become uncoupled and phosphorylation was more seriously affected than oxygen consumption. They also reported that, under these conditions, the cytochrome c content of the medium increased which they interpreted as a result of damage to the mitochondrial membrane.

Kallapur, Downer, George and Thompson (1982) studied the effect of temperature on the phase properties and lipid composition of flight muscle mitochondria of *Schistocerca gregaria*, they observed that elevated temperatures resulted in depressed levels of some phospholipids and they suggested that it is possible that some of the observed effects of temperature on insect metabolism may result from temperature-induced alterations in mitochondrial membranes.

O'Brien, Dahlhoff and Somero (1991) have also reported that incubation at elevated temperatures caused inactivation of mitochondrial respiration and caused the activities of several mitochondrial enzymes either to increase or decrease sharply in the hydrothermal vent tube worm *Riftia pachyptila.*

When isolated mitochondria are provided with a substrate and phosphate in an oxygenated medium, the esterification of ADP to ATP is coupled to oxygen consumption, and the ADP:O ratio gives a direct measurement of coupling efficiency. Since mitochondria are the most important sites of energy production in aerobic cells, any damage to this system is likely to have a serious effect upon the level of ATP and

other high energy molecules which may lead to the impairment of energy requiring cell processes, and the consequent breakdown of cellular metabolism.

In this study the mitochondria of the flight muscle of *Calliphora vicina* have been used. These mitochondria are easily accessible and isolation time is relatively short. However, in some respects mitochondria are unsatisfactory for their morphological integrity and functional behaviour can be altered during isolation (Balboni, 1965; Carney, 1966); structural damage occurring during isolation will markedly affect coupling (Van den Bergh, 1962). Sarcosomes can be "aged" by grinding during isolation or by leaving them for long periods of time after isolation (Lewis and Slater, 1954; Van den Bergh, 1962). However, paying attention to these facts and using gentle isolation procedures, good mitochondria whose coupling capacities approach theoretical values can be isolated (Van den Bergh, 1962). Furthermore, ensuring standardisation of isolation procedure, intact sarcosomes can be obtained, which are satisfactory for use in comparative physiological studies.

Earlier work on heat injury and death in blow flies has shown a good correlation in *Calliphora erythrocephala* with the loss of flight ability (Davison and Bowler, 1971). They presented data that showed that the mitochondria from flight muscle had a thermal sensitivity equivalent to that of the intact organism. Furthermore Davison and Bowler (1971) and Bowler and Kashmeery (1981) also reported that *in vivo* (LD_{50}) heat treatment of blowflies caused impairment in the functional efficiency of sarcosomal respiration when glycerol-3-phosphate was the substrate. The mitochondria from heated flies had poor respiratory control and in many instances ADP:O ratios could not be demonstrated. This work satisfies one of the criteria for a primary lesion in that, a very close correlation existed between the age-specific heat sensitivity of the flies and the thermal damage caused to mitochondria by a particular heat dose. In a

more complete study on the recovery of blowflies from sublethal heat injury Bowler and Kashmeery (1981) using the time for the return of normal mitochondrial function as the index of repair, have shown that the repair itself is temperature sensitive.

The results from the previous chapter show that adult heat death points were changed by pretreatment (heat shock). The question, therefore arose whether sarcosomal oxidative phosphorylation would be protected from impairment during the development of thermotolerance following heat shock.

4.2 MATERIALS AND METHODS.

Male and female 10-day old flies of Durham stocks used in this study were developmentally acclimatised to 24 ± 0.5°C and the heat treatments were given using the methods described in chapter 3.

Flight muscle mitochondria were isolated after the procedure Tribe and Bowler (1968); Davison and Bowler (1971) and Bowler and Kashmeery (1981).

4. 2.1 Preparation of mitochondria.

Thirty to forty 10-day old flies were immobilised with carbon dioxide. Thoraces were transferred to a small glass cold, loosely fitting, flat-bottomed glass rod. Care was taken to avoid twisting or grinding the rod, and so damaging the sarcosomes. The resulting pulp was transferred with a further 1 cm^3 of sucrose medium I. The pulp squeezed through the muslin by hand and the filtrate was collected in a centrifuge tube on ice. The filtrate was centrifuged at 4°C in an Europa 24 centrifuge at 6000 g for 10 min. The resulting pellet was suspended in 2 cm^3 of ice cold sucrose medium II. It was then recentrifuged at 6000g for another 10 minutes and the final pellet of washed mitochondria suspended in 0.5 cm^3 of resuspension medium of III. The average time for the whole preparation period was approximately 50 min.

Twenty separate preparations were made for each of the control, LD_{50} treated control and LD_{50} treated thermotolerant flies.

Isolation media used:

(i) Isolation medium I

0.32 M sucrose, 10 mM EDTA, 2% BSA, 10 mM Tris/HCl, buffered at pH 7.3

(ii) Isolation medium II

As sucrose medium I without the BSA

(iii) Isolation medium III

0.15 M KCl, 1 mM EDTA, 10 mM Tris/HCl buffered at pH 7.3

4.2.2 Measurements of oxidative phosphorylation

Oxidative phosphorylation was measured at 24°C (unless stated otherwise) using the Clark oxygen electrode (Clark, 1956). The reaction medium was the same as that used by Davison (1969) and Kashmeery (1979) and was:

50 mM KCl, 30 mM phosphate buffer (Sorensen) pH. 7.3, 5 mM $MgCl_2$ $6H_2O$, 1 mM EDTA, 20 mM Tris/HCl buffered at pH 7.3

The medium was thoroughly equilibrated to 24°C for several hours to saturate it with atmospheric oxygen.

4.2.3 Description of the oxygen electrode

The electrode has a perspex reaction vessel surrounded by a water jacket and this unit screws onto a perspex base where a platinum cathode and silver anode are situated. When in use both electrodes are covered with a few drops of 1 M KCl and this electrolyte is separated from the reaction vessel by a teflon membrane. The perspex screw cap fitted to the top of the reaction vessel had a small hole bored through the centre to allow additions to be made to the reaction media. The medium in the reaction vessel was continuously stirred using a small magnetic stirrer and a "flea".

The principle of the oxygen electrode has been described by Davies & Brink (1943). When a polarising voltage of -0.6 volts is imposed across the two electrodes, the platinum negative to the silver, oxygen undergoes an electrolytic reduction

$$O_2 + 2e + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + 2e + 2H^+ \rightarrow 2\ H_2O$$

and the current flowing is directly proportional to the oxygen content of the medium. Chappell (1964) has pointed out that the Clark electrode measures activity and not the concentration of the oxygen present in the reaction medium. Therefore in this study the electrode has been calibrated by determining the oxygen content of the reaction medium (Davison, 1970).

The current flowing through the electrode was passed through a helical potentiometer in series with the platinum electrode and the voltage developed across this resistance was fed into a recorder (Goerz Electro) set at 2 mV sensitivity. The recorder baseline was set by reducing the medium in the reaction vessel of the oxygen electrode with sodium dithionite. This effectively reduced the current across the electrode to zero. Reaction medium saturated with oxygen was pipetted into the reaction vessel and the potential adjusted with the helical potentiometer to give a full scale deflection on the recorder.

4.3.4 Polarographic measurement of oxygen consumption

3 cm^3 of reaction medium was pipetted into the reaction chamber of the oxygen electrode, the perspex screw cap was replaced such that all air bubbles were expelled. The surface of the reaction medium was allowed to rise about 1 mm up the central hole of the stopper to minimise the contact between the reaction medium and atmospheric

oxygen. After about one or two minutes the recorder was adjusted to give a full scale deflection of the pen recorder. Oxygen uptake from this 3 cm^3 of reaction medium was followed by determining the change of deflection of the recorder after the following additions:

50 µl of 2 M glycerol-3-phosphate solution were added (final concentration 33 mM) and oxygen consumption was followed for about one minute to give the substrate rate respiration. Then 10 µl of 50 mM ADP (in 30 mM phosphate buffer at pH 6.8) was added and the ADP-stimulated respiration (state III) was observed after this addition. At the expenditure of added ADP State IV respiration was then followed for up to one minute. The experiments was then repeated with a fresh mitochondrial sample but with the addition of 50µl of 1M pyruvate + proline (final concentration, 2 mM each).

Endogenous rates were negligible. Measurements of oxidative phosphorylation were repeated several times, but not later than 1 hour after isolation, for results after this time became inconsistent, indicating sarcosomal ageing. Respiratory rates were determined from the slopes of the polarographic traces as indicated by the construction lines in Figure 4.2, trace 1.Respiratory rates were expressed as µg AO mg protein $^{-1}h^{-1}$ see Figures (4.2 and 4.3).

Respiratory Control Index (RCI)

For polarographic studies Chance and Williams (1955) have defined the respiratory control index as the ratio of the respiratory rate in the presence of added ADP to the rate following its expenditure i.e.:

$$RCI = \frac{StateIII}{StateIV}$$

However, in some experimental conditions e.g. following heat treatment to the whole animal, no respiratory cut-off occurred after the addition of ADP, consequently it was not possible to measure RCI according to the Chance and Williams definition.

ADP:O ratio:

The ADP:O ratio is the number of moles of ADP esterified for each gram atom of oxygen consumed. When pyruvate is the substrate, electrons are donated to the electron transport chain before the flavine nucleotides and consequently there are 3 phosphorylations before the reduction of oxygen (ADP:O = 3). Glycerol-3-phosphate donates electrons via a specific dehydrogenase and electrons enter the electron transport chain after the flavine nucleotides so that only two phosphorylations occur with this substrates (ADP:O = 2).

The ADP:O ratios have been calculated throughout this study using the method described by Chance & Williams (1955). When a known amount of ADP is added to a mitochondrial suspension in the presence of substrate, the respiration rate increases and a quantity of oxygen is consumed. The uptake of oxygen can be measured from the trace by extrapolating the ADP- stimulated rate and the rate after its expenditure. The distance between the point of addition of ADP and the extrapolated point of its extinction, represents the quantity of oxygen required by respiration, when 0.5 μmoles of ADP were converted to ATP.

Figure 4.2 shows a typical trace indicating how state III and state IV respiration were determined and how RCI and ADP:O ratios were calculated.

4.2.5 Determination of sarcosomal protein:

The concentration of sarcosomal protein was determined using the Coomassie brilliant Blue-G250 method described by Bradford (1976), BSA fraction V was used as a standard.

Protein reagents:

0.01% (W\V) Coomassie Blue G250

47% ethanol

8.5 % phosphoric acid

A 200 µg / ml solution of BSA was prepared, this was diluted to give solutions within the range 40- 200 µg / ml. A typical calibration curve is shown in Figure 4.1.

Procedure:

The unknown samples taken from the sarcosomal isolations were dissolved in 1 cm^3 0.1M NaOH. 100 µl of each sample was taken, and 100 µl of 1.1 M NaOH added. These were mixed, 5 ml of Coomassie blue was then added to each sample, mixed and allowed to stand for 15 minutes at room temperature. The solutions were poured into a glass cuvette, and the absorbancy was measured against water at 595 nm in a LKB Biochrom. Ultraspectrophotometer.

From the standard solutions prepared, a calibration curve of absorbance against protein concentration was plotted, Figure 4.1 and the unknowns were determined from this curve.

4 .2.6 Chemicals

The chemical reagents used in the present study were AnalaR grade where possible, or were of the highest purity which could be obtained commercially. Solutions were made up in glass distilled water.

Adenosine -5'-diphosphate (disodium salt), sodium pyruvate, proline, EDTA, bovine serum albumin, rac Glycerol 3-phosphate (sodium salt) were purchased from Sigma Chemical Co., Ltd.

ADP was stored as a frozen solution buffered at pH 6.8, at -20°C for a maximum of six weeks.

4.2.7 Statistical treatments:

All values are presented as means ±S.E.M. Student's *t*-tests or the Mann- Whitney *U*-test were used to determine the levels of significance as appropriate.

4.3 RESULTS:

A typical electrode recording for control (not LD_{50} treated), LD_{50} treated and LD_{50} treated thermotolerant flies are presented in Figures 4.2 and 4.3 using both glycerol-3-phosphate and pyruvate plus proline as substrates.

It should be emphasised that the traces obtained for the two substrates were from different samples of the same mitochondrial preparations. Furthermore, in all cases mitochondrial respiratory performance was measured at 24°C.

4.3.1 Glycerol 3-phosphate as substrate

(i) Non pretreated (control flies):

The QO_2 values for endogenous respiration (without added substrate) were usually not measurable. The addition of substrate glycerol-3-phosphate caused an immediate rise in respiratory rate, as indicated by downward deflection of the oxygen trace, and this was further stimulated by the addition of 0.5 μmoles of ADP, which initiated state III respiration, and after the utilisation of the added ADP, the rate of respiration decreased and state IV respiration was obtained. Further addition of ADP produced the same effect until the oxygen in the reaction medium was exhausted. From these traces respiratory rates, ADP:O ratio and respiratory control indices (state III / state IV) were estimated. The respiratory rates observed with these non-pretreated control mitochondria are recorded in Table 4.1.

The substrate rate oxidation was 35.93 ± 0.57 μg A0 mg protein^{-1}h^{-1}. This respiration was stimulated by the addition of ADP to give the QO_2 value of 81.83 ± 2.53 μg AO mg protein^{-1}h^{-1} (The ADP-stimulated rate). After the expenditure of the added ADP, the rate of respiration decreased to give the value of 37.45 ± 2.03 μg AO

mg protein^{-1} h^{-1} that is state IV. This resulted in obtaining good values for RCI (mean = 2.18) and mean ADP:O of approximately 2 as expected, this implies a high level of oxidation and phosphorylation.

(ii) Non-pretreated LD_{50} flies

The mitochondria isolated from flies after an LD_{50} heat treatment have impaired respiratory performance. Substrate rate respiration was significantly lower than that of control (non-pretreated) mitochondria ($P < 0.001$), it was approximately half the value of that of control mitochondria as it fell from 35.93±0.5 to 22.1 ± 1.67 µg AO mg protein $^{-1}$ h^{-1} , this suggested that mitochondrial electron transport is inhibited. The ADP-stimulated rate (state III) respiration rate showed a decline of about 75% and was significantly reduced from 81.83 ± 2.53 in the control group to only 22.21 ± 1.67 µg AO mg protein^{-1} h^{-1} ($P < 0.001$). State IV respiration was also reduced, it fell from 37.45 ± 2.03°C in the control group to only 22.21 ± 1. 67 µg AO mg protein^{-1} h^{-1} ($P < 0.001$). The state III respiration in these mitochondria was the same as state IV respiration. In such mitochondria the ADP stimulation was not demonstrable, hence an RCI value of one was obtained, and consequently ADP:O was not measurable. The results obtained are entirely consistent with data produced by other workers on flight muscle mitochondria. Moreover, these results suggest that LD_{50} heat treatment markedly affect oxidative phosphorylation using glycerol-3-phosphate as substrate.

(iii) Thermotolerant LD_{50} flies

Generally these mitochondria have a respiratory performance similar to that of non pretreated control mitochondria, the improvement over non-thermotolerant LD_{50} control flies was pronounced when glycerol-3-phosphate is the substrate.

The substrate respiration (before the addition of ADP) showed a trend towards a significant decline as compared with the control non-pretreated flies, It fell from 35.93 ± 0.57 (in the non-pretreated control) to 27.23 ± 1.74 μg AO mg protein^{-1} h^{-1} in these thermotolerant control flies. However, it is still significantly higher than the LD_{50} control mitochondria that is 22.1 ± 1.67 μg AO mg protein^{-1} h^{-1} ($P < 0.001$).The state III respiration rate in these thermotolerant control flies being 70.48 ± 2.12 μg AO mg protein^{-1} h^{-1} is significantly higher as compared with that of the LD_{50} control group which is 22.21 ± 1.67 μg AO mg protein^{-1} h^{-1}. However, it is still significantly lower than that of the control group 81.83±2.53 ($P < 0.001$). State IV respiration rate which is 32.78 ± 1.68 μg AO mg protein^{-1}h^{-1} is similar to that of non-pretreated control mitochondria, but was significantly higher than that of 22.21 ± 1.67 μg AO mg protein^{-1} h^{-1} found for mitochondria of the LD_{50} control group ($P < 0.001$). The RCI value is not different to that of non-pretreated control, but it rose from 1 in LD_{50} control flies to 2.22 ± 0.07 in these mitochondria of thermotolerant control flies. Measurable ADP:O values were obtained from mitochondria from LD_{50} treated thermotolerant flies, however, they were significantly lower than those of non-pretreated control mitochondria ($P < 0.001$).

Pyruvate plus proline as substrates

(i) Non pretreated control flies:

The results of measurement of oxidative phosphorylation of mitochondria from control flies are also recorded in Table 4.1. Generally when pyruvate plus proline were used as substrates QO_2 values were lower than with glycerol-3-phosphate. The QO_2 value obtained for substrate respiration is 4.8 ± 0.19 µg AO mg protein^{-1} h^{-1}. This respiration was stimulated by the addition of ADP. The state III respiration obtained was 50.19 ± 2.62 µg AO mg protein^{-1} h^{-1} and after the exhaustion of this added ADP, state IV respiration had fallen to 8.75 ± 1.09 µg AO mg protein^{-1} h^{-1}. These data gave a high mean value for the RCI of 5.75 but the mean value for ADP:O ratio was found to be 2.75 which in close to the expected value.

(ii) Non-pretreated LD_{50} flies:

As compared with non pretreated controls substrate rate respiration was significantly increased ($P < 0.001$) in mitochondria following an LD_{50} treatment. The QO_2 value rose from 4.8 ± 0.18 in the controls to 6.98 ± 0.18 µg AO mg protein^{-1} h^{-1} in the LD_{50} treated mitochondria. The state III respiration rate, however, was significantly reduced from 50.19 ± 2.62 to 23.99 ± 1.63 µg AO mg protein^{-1} h^{-1} ($P < 0.001$), but state IV respiration showed only a small but significant increase over the non pretreated control mitochondria ($P < 0.05$) rising from 8.75 ± 1.09 to 12.77 ± 1.54 µg AO mg protein^{-1} h^{-1}. The increased state IV respiration and the reduction in state III respiration, caused a sharp reduction in RCI from 5.75 in the non pretreated controls to 1.93 ($P < 0.001$). The ADP:O ratios were still measurable, although a reduction in their values was observed ($P < 0.01$). This is in agreement with the preliminary results reported by Davison (1969). Thus pyruvate plus proline supported

respiration is less sensitive to *in vivo* LD_{50} heating than that supported by glycerol 3-phosphate.

(iii) Thermotolerant LD_{50} flies

In these mitochondria we can note a significant protection from heat damage has occurred, see Table 4.1. The substrate rate respiration is significantly increased compared with the control LD_{50} treated mitochondria. It rose from 6.98 ± 0.18 to 7.38 ± 0.2 μg AO mg protein^{-1}h^{-1}. However, state III respiration remained significantly lower in these mitochondria as compared with control LD_{50} non-pretreated mitochondria, the QO_2 was calculated to be only 23.99 ± 1.27 μg AO mg protein^{-1} h^{-1} ($P < 0.001$). However, state IV respiration was restored to the lower values obtained for non-pretreated control mitochondria. Values calculated for RCI correspondedly increased from 1.93 ± 0.16 to 3.62 ± 0. 25 in thermotolerant mitochondria, the latter value was still significantly lower than that obtained for non-pretreated control mitochondria ($P < 0.001$), but higher than for non-pretreated LD_{50} mitochondria. Once again, however ADP:O ratios were less affected and a mean of 2.70 ± 0.08 was obtained which did not differ from non-pretreated control values.

In contrast to the use of glycerol-3-phosphate, as a substrate ADP:O ratios were measurable in mitochondria from all treatment groups using proline plus pyruvate substrate, see Table 4.1.

Thus the indices of the quality of function of the isolated mitochondria (RCI and ADP:O) were both significantly reduced by exposing the flies to an LD_{50} treatment, showing that oxidative phosphorylation was impaired. This damage is more pronounced when glycerol-3-phosphate was the substrate than with pyruvate plus proline. Table 4.1 shows that pretreatment with a heat shock that induces

thermotolerance, protected the mitochondria from damage caused by the subsequent LD_{50} dose, because the values obtained for RCI and ADP:O, with both substrates conditions, were restored closer to the values from control mitochondria. This raised the question whether the induction of thermotolerance *in vivo* will protect mitochondrial respiratory function against an *in vitro* exposure to high measuring temperature.

Figure 4.1. Typical calibration curve for protein using Coomassie Brilliant Blue-reagent Bradford (1976). BSA (fraction V) was used as standard.

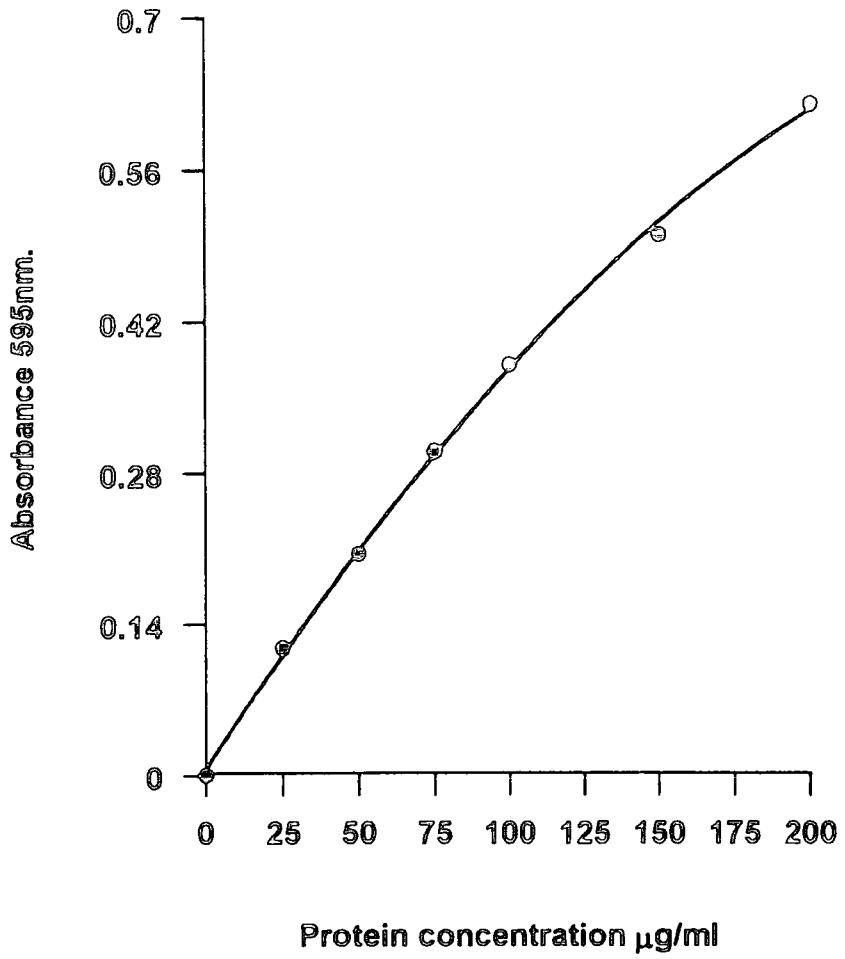

0.7
0.56
0.42
0.28
0.14
0
Absorbance 595nm.
0
25
50
75
100
125
150
175
200
Protein concentration µg/ml

Table 4.1. The effect of *in vivo* LD_{50} treatment on the respiratory efficiency at 24°C of mitochondria from normal and thermotolerant blowfly flight muscle.

Respiratory rate is measured in μg AO mg protein^{-1} h^{-1}. The mean value for LD_{50} and LD_{50} thermotolerant groups were compared with those from control (none) mitochondria. Statistical differences were derived using Students's t-test or Whitney-Mann U-test as appropriate; * $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

N = 20 in all cases.

RCI = State III / State IV (see Figure 4.2).

ADP:O is the ratio of the number of moles of ADP esterified to ATP for each gram of added ADP.

NM = non measurable.

Substrate	Treatment	RESPIRATORY RATE						
		Endogenous (without substrate)	With substrate	State III	State IV	RCI	ADP:O	Protein concentration (mg 50 µl)
Glycerol 3-phosphate	None	1.27±0.06	35.93±0.57	81.83±2.53	37.45±2.03	2.18±0.1	1.92±0.06	0.32±0.02
	LD_{50} control	N.M.	22.1±1.67***	22.21±1.67***	22.21±1.67***	1	N.M.	0.28±0.02
	LD_{50} thermo-tolerant	1.02±0.2	27.23±1.74***	70.48±2.12***	32.78±1.68	2.22±0.07	1.69±0.03***	0.31±0.02
Pyruvate + proline	None	N.M.	4.8±0.18	50.19±2.62	8.75±1.09	5.75±0.13	2.94±0.08	0.32±0.02
	LD_{50} control	N.M.	6.98±0.18***	23.99±1.83***	12.77±1.54*	1.93±0.16***	2.60±0.1**	0.28±0.02
	LD_{50} thermo-tolerant	N.M.	7.38±0.2***	23.99±1.27***	6.72±0.94	3.62±0.25***	2.70±0.08	0.31±0.02

Figure 4.2 Typical polarographic records obtained showing the rates of oxygen consumption of isolated flight muscle mitochondria at 24°C with glycerol-3-phosphate as substrate.

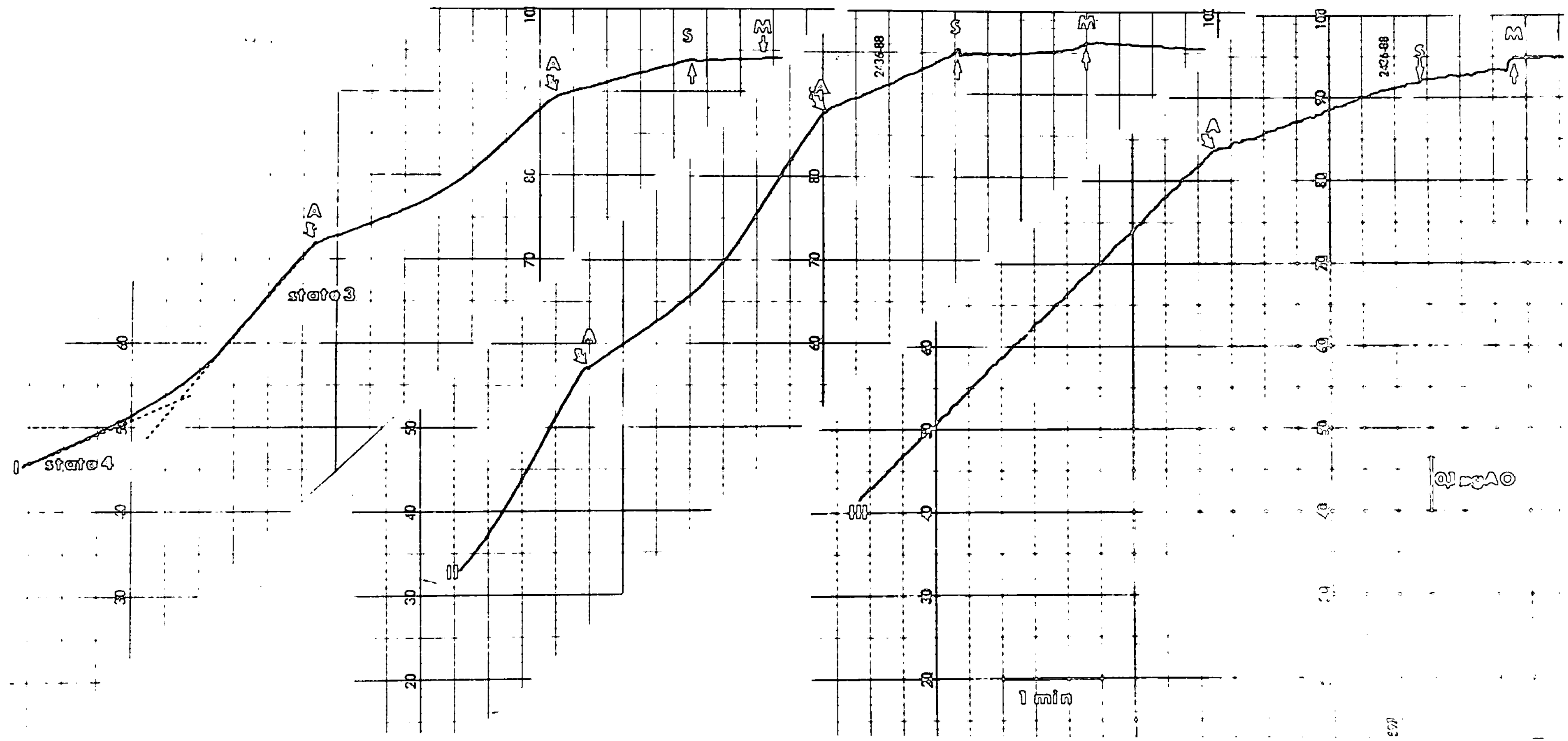
Arrows indicate additions of mitochondria (M) , substrate (S) and 0.5μmole of ADP.

Trace I shows results from mitochondria from control flies

Trace II shows results from mitochondria isolated from thermotolerant flies after *in vivo* LD_{50} dose.

Trace III shows results from mitochondria isolated from control flies after an LD_{50} dose.

The method of estimation of State III and State IV respiration is shown from which RCI and ADP:O can be calculated (Chance and Williams, 1955)



I
II
III
stato 3
stato 4
A
S
M
2436-88
1 min

Figure 4.3. Typical polarographic records obtained showing the rates of oxygen consumption of isolated flight muscle mitochondria at 24 °C with pyruvate plus proline as substrate .

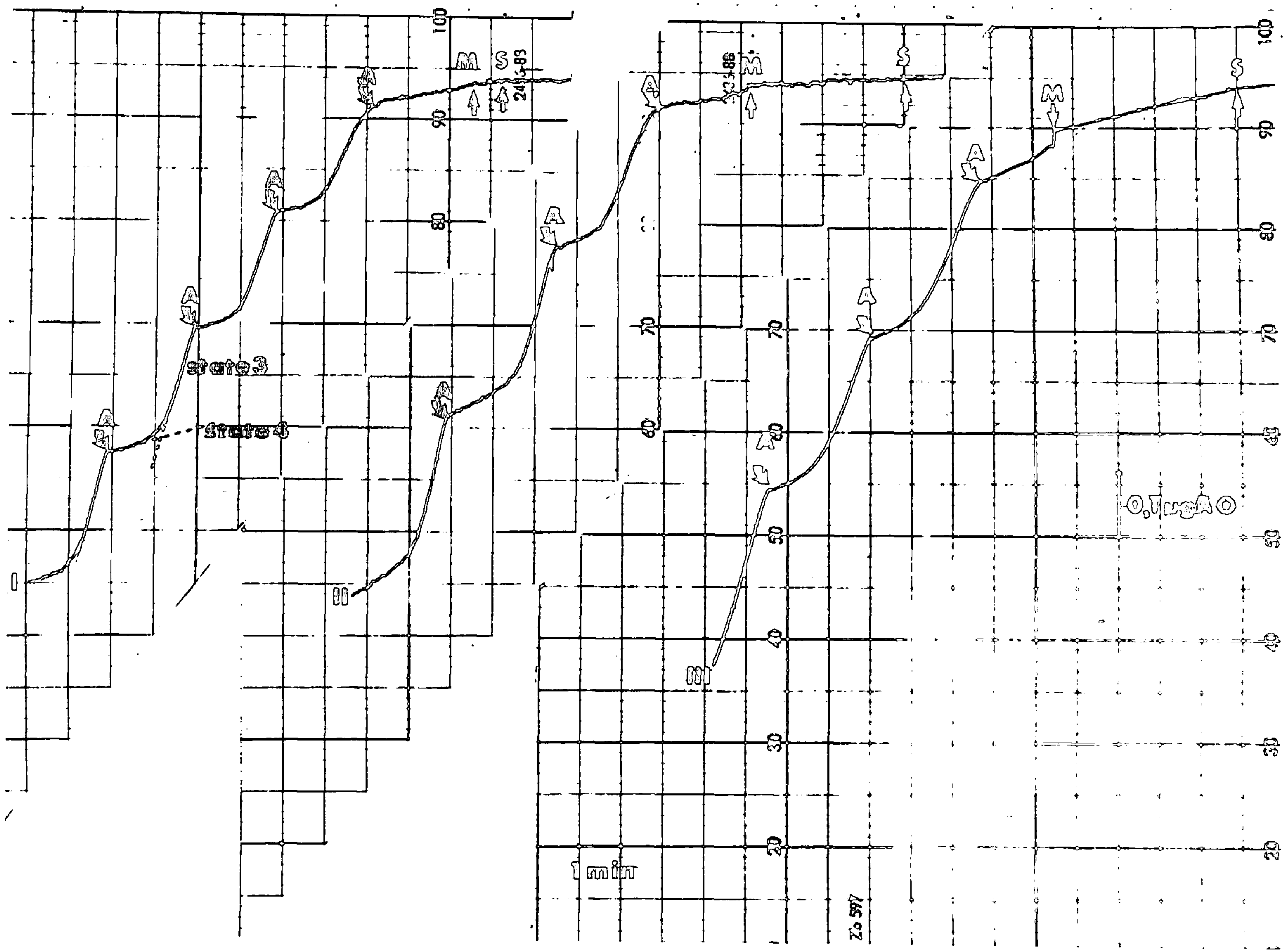
Arrows indicate additions of mitochondria (M), Substrate (S) and 0.5 μmol of ADP

Trace I shows results from mitochondria from control flies

Trace II shows results from mitochondria isolated from thermotolerant flies after *in vivo* LD_{50} dose.

Trace III shows results from control mitochondria from control flies after an LD_{50} dose.

The method of estimation of State III and State IV respiration is shown from which RCI and ADP:O can be calculated (Chance& Williams, 1955).



state 3
state 4
1 min
Zo 597

4.4 DISCUSSION:

In this study, and in studies by others (for example Danks and Tribe, 1979) state III and state IV respiration rates after the second addition of ADP have been shown to be higher than after the first addition. It is therefore questionable whether all state III or all state IV respiration rates should be grouped to give a mean value, and in the present study means were only obtained from the first addition of ADP. However, it should be emphasised that different aliquots of the same mitochondrial suspension were used for both substrates glycerol-3-phosphate and pyruvate plus proline. Therefore the data obtained for the two substrates can be compared.

The functional performance of the mitochondria from this stock of flies was very similar to that obtained in earlier studies (Davison and Bowler, 1971; Bowler and Kashmeery, 1981). What is also significant is that, notwithstanding the difference in LD_{50} (see chapter 3) the data obtained for flies given an LD_{50} treatment was also effectively the same (Bowler and Kashmeery, 1981). Glycerol-3-phosphate respiration was found to be differentially sensitive to *in vivo* heating as compared to respiration using pyruvate plus proline. Respiratory control was lost with glycerol-3-phosphate respiration, but in the same mitochondrial sample, respiratory control although reduced, was still demonstrable, when pyruvate plus proline were used as the substrate. Furthermore, ADP:O also shows a significant if less dramatic fall with pyruvate plus proline respiration. The reduction in state III respiration with both substrates however indicates that either the respiratory chain was inhibited by heating *in vivo* and/or phosphorylation was inhibited with oxidative phosphorylation remaining coupled. In the case of glycerol-3-phosphate there was no evidence of uncoupling of oxidation and phosphorylation, because there was no increase in state IV respiration. However, with pyruvate plus proline as substrate some uncoupling is suggested because State IV

respiration is significantly increased in mitochondria isolated from LD_{50} non pretreated flies.

A similar differential sensitivity of these two respiratory pathways has been reported by other workers, Van den Berg and Slater (1962) and Tribe and Ashhurst (1972). The differential response of mitochondria from heated flies to the different substrates makes the identification of the primary lesion a problem. It could be argued, as proposed by Davison and Bowler (1971), that the loss of respiratory control and the reduction in State IV respiration, and together with the inhibition of State III respiration, resulted from heat damage to the ATP synthesising enzymes, without any uncoupling of oxidation and phosphorylation.

Floridini *et al.* (1987) have shown in mitochondria isolated from hyperthermia-treated Ehrlich ascites tumour cells that State III and State IV respiration were both decreased, whilst RCI and phosphorylation remained normal. Their work suggested a general inhibition of the electron transport chain because heating affected different segments of the respiratory chain at all energy conserving sites. A reduction in ATP synthesis would also be predicted because Lunec and Cresswell (1983) reported that heating S5178YS cells resulted in a rapid reduction in ATP levels, a result confirmed by Jaing *et al.,* (1991) from ^{31}P NMR studies.

The induction of thermal tolerance afforded significant protection to the mitochondria during a subsequent LD_{50} heating. In the case of glycerol-3-phosphate respiration, respiratory control was restored to control levels, although ADP:O remained significantly lower. State III respiration was restored to about 90% of that of controls. In the case of pyruvate plus proline respiration the protection afforded was to reduce State IV respiration, rather than increase State III respiration, which remained inhibited, this suggested that the protection served to prevent uncoupling. Lunec and Cresswell (1983)

have also shown that the development of thermotolerance in L51178YS cells also protected the ability to maintain ATP levels in cells subjected to a second heat treatment, a response that depended upon the time course of the appearance and the decay of thermotolerance. The reason for the observed differential effect on heat on respiration using the two different substrates, with the same mitochondrial sample, is not clear, (Bowler and Kashmeery, 1981). Glycerol phosphate dehydrogenase is thought to be the rate limiting step for glycerol phosphate oxidation by the respiratory chain, (Lardy *et al.*, 1962), this enzyme is allosterically stimulated by 10^{-5} g. ionl^{-1} Ca^{2+} (Hansford and Chappell, 1967). In an earlier work it was shown that whilst the catalytic properties of the enzyme are affected, V_{max} was reduced by 50%) in the LD_{50} treated mitochondria, its allosteric properties (Hill exponent) were not significantly affected. Furthermore the enzyme was shown to be relatively thermostable (Bowler and Kashmeery, 1981). Several earlier studies indicated that, in mitochondria from other sources, a temperature sensitive site exists in the vicinity of coenzyme Q and site II (Morris and King, 1962; Christiansen and Kvamme, 1969) which might account for the particular sensitivity of glycerol-3-phosphate respiration in blowfly flight muscle mitochondria which have a high capacity for respiration using this substrate. It is clear that phosphorylation is impaired by *in vivo* heating, for there is a significant reduction in respiratory control, with both substrates, without a rise in state IV respiration to the level obtained for state III respiration in control mitochondria, which would be expected if oxidative phosphorylation was uncoupled, Table 4.1. O'Brien *et al.* (1991) reported that a number of mitochondrial oxidative enzymes, from different species, were inactivated by heat in a way that parallels the differences in organism body temperature. Treatment of the preparations with chaotropic agents, that disrupt hydrophobic interactions between membrane lipids and proteins, markedly increased the thermal sensitivity of the proteins. These authors

concluded that this suggested that thermal inactivation of mitochondrial function results from the perturbation of the hydrophobic interactions between lipids and proteins, a conclusion which is in agreement with the current view that membranes are a primary target in cellular heat injury (Bowler *et al.,* 1973; Bowler, 1987; Bowler and Manning, 1994). This work also showed that membrane dependent processes are susceptible to thermal damage and are sensitive in the same temperature range as that which impairs function in the intact organism.

CHAPTER V

THE EFFECT OF MEASURING TEMPERATURE ON RESPIRATORY PERFORMANCE, *IN VITRO*, OF MITOCHONDRIA FROM CONTROL AND THERMOTOLERANT BLOWFLIES.

5.1 INTRODUCTION

For ectotherms the maintenance of physiological function over a range of body temperature requires seasonal, even daily, adjustment in both the rates of physiological processes and the biochemical composition of the organism (Hochachka and Somero, 1984; Hazel and Williams, 1990). However, acclimatory ability has its limits in all species and no one species has been discovered that is able to remain metabolically active over the entire range of environmental temperature that may be experienced (Cossins and Bowler; 1987, Prosser and Heath, 1991).

A large literature exists demonstrating temperature dependence of respiration in many insect species, indeed Keister and Buck in their 1964 review, reflect that this is perhaps the most over demonstrated phenomenon in insect physiology. In most studies the respiratory rate (per individual or per unit rate) classically depends on temperature and is low at low temperatures and increases rapidly through the midrange temperatures, but then breaks sharply as lethal temperatures are approached. Such data have been recorded for one or more stages of *Calliphora* (Davison, 1970), *Phormia* (Keister and Buck, 1961), *Galleria* (Burkett, 1962), *Periplaneta* and *Blaberus* (Wojtczak, et al., 1968), *Musca* and *Protophormia* (Wood and Nardin, 1980), *Rana* (Feder, 1982), *Desmognathus* (Feder, Gibbs, Griffin and Tsuji, 1984).

The effect of experimental temperature on mitochondria has been followed by many workers. Frequently, such studies have only concerned the determination of oxidation rates, with the data presented as Arrhenius curves, e.g. Lyons & Raison (1970), Smith (1973c). However, when oxidative phosphorylation is strongly coupled, oxidation rates will be limited by the availability of ADP, as can be already seen in Figures 4.2 and 4.3, and may not respond fully to changes in temperature. As a case in point adenine nucleotide translocase is reported to be very sensitive to low temperature

Heldt & Klingenberg (1968). This enzyme exchanges cytosolic ADP for mitochondrial ATP, so supplying the mitochondrial ADP pool. In fact this translocation process has been reported to be rate limiting in oxidative phosphorylation in rat liver mitochondria in the temperature range 0-23°C (Kemp, Groot and Reistma, 1969). In consequence, the temperature sensitivity of oxidation rates in coupled mitochondria is likely to reflect the effect of temperature on processes that supply ADP to the mitochondrial pool, rather than an affect on the activity of respiratory enzymes.

An interesting feature of Arrhenius plots of oxidation rates of mitochondria is that, in most cases, breaks, or discontinuities occur. However, some differences of opinion are held concerning the causes of, and therefore interpretation of 'breaks' in Arrhenius curves. The breaks have been interpreted as sudden transitions from one rate limiting step to another, each with quite different values for activation energy. Other, more contrasting views argued that such interpretations are unlikely to hold for multienzyme reactions such as occur in respiration, which also involves physical processes, such as diffusion, (see Cossins and Bowler, 1987 for a fuller discussion).

Newell & Walkey, (1966) and later Newell & Pye (1971) reported a temperature-independent plateau for respiration of mitochondria from a variety of species. The dominant role of mitochondria in cellular respiration had led Newell and his co-workers to seek an explanation, at the biochemical level, of temperature independent metabolism they had demonstrated in intertidal animals (Newell and Pye, 1971). Such an explanation was necessary for *a priori* reasoning would suggest metabolic rate should obey the Arrhenius law. This mitochondrial work of Newell and Walkey (1966) has also been criticised by Tribe and Bowler (1968) and Davies and Tribe (1969) not only in the choice of inappropriate substrates, but also the use of atypical isolation procedures, furthermore, in no case was the functional state of the isolated

mitochondria determined. Davies and Tribe (1969) have investigated the effect of temperature upon the oxygen consumption of intact poikilotherms and their tissues, and also of mitochondria from poikilotherms and a homeotherm. Their investigation also included preparations of flight muscle tissue and mitochondria from *Calliphorara erythrocephala*. They concluded that in neither the intact animal at rest, nor endogenous oxygen uptake by isolated cells, and tissues was there evidence of a plateau of temperature independent respiration as suggested by Newell (1966).

Indeed, work on mitochondria, from a variety of tissue and organism sources, show respiration to be very sensitive to temperature (Kemp *et al.*, 1969; Smith, 1973c and Lee and Gear, 1974) and adenine nucleotide translocation (Heldt and Klingenberg, 1968). This earlier work provided a basis for subsequent studies of the effect of temperature on mitochondria. Most notable is the work by Raison on the influence of temperature on respiratory enzymes (Raison, 1973). These investigations were carried out on mitochondria isolated from homeothermic and poikilothermic animals (Lyons and Raison, 1970, Kumamoto, Raison and Lyons, 1971) measuring State III and State IV of succinate oxidation. The Arrhenius plots showed a discontinuity in both State III and State IV respiration at 24°C in homeothermic animals whereas the relationship was linear for poikilothermic animals. From spin-label studies Raison and his co-workers concluded that the cause of the sharp change in activation energy (E_a) was a consequence of a temperature-induced phase change in membrane lipids (Raison *et al* 1971). Such a phase change was proposed to affect the rate of functioning of the members of the electron transport chain. Similar studies were also carried out by Smith (1973c) on mitochondria from fish liver tissue, and confirmed that both State III and State IV gave a linear plot, but non-linear plots were obtained with mammalian mitochondria (Smith, 1973c).

The effects of elevated temperatures on mitochondrial respiration and the activities of mitochondrial enzymes differ among species in accordance with their differences in their maximal body temperatures as shown in the recent study of (Dahlhoff, O'Brien, Somero and Vetter, 1991) using acute exposure times, they showed that mitochondrial respiration and the activities of the membrane associated enzymes of mitochondria were inactivated by temperatures that exceed the upper habitat temperature of the species by approximately 10-20°C. In a similar study O'Brien, Dahlhoff and Somero, (1991) examined the causes of thermal disruption of mitochondrial respiration, the method used in their study was designed to examine the irreversible or long-term effects of high temperature on mitochondrial function, i.e. they measured the rates of succinate-supported respiration and activities of cytochrome c oxidise, succinate dehydrogenase and malate dehydrogenase in mitochondria from hydrothermal vent tube worm *Riftia pachytila* and the coastal bivalve *Solemya reidi.* Their results suggested that loss of respiratory activity of intact mitochondria, and large changes in activities of membrane-associated mitochondrial enzymes at elevated temperatures may be a consequence of disruption of hydrophobic interactions in the membrane-protein and their closely associated lipids, or between proteins themselves.

More recently Dahlhoff and Somero (1993) studied the effect of temperature on mitochondrial oxygen consumption, membrane fluidity and cytochrome c oxidase activity of five species of eastern Pacific Abalone. Their results showed that the temperature at which Arrhenius plots of respiration rate of mitochondria from freshly collected Abalone exhibited sharp breaks in slope correlated with the habitat temperature at the time of capture of each species.

In a comparative study on fishes Johnston *et al.* (1994) have investigated the thermal tolerance and the respiratory properties of isolated red muscle mitochondria in

the fish *Oreochromis grahami*, from the alkaline hot-springs, Lake Megadi, Kenya where water ranged in temperature from 30 to 40°C. In order to assess evolutionary temperature adaptation of maximal mitochondrial oxidative capacities, the rates of pyruvate and palmitoyl carnitine utilisation in red muscle mitochondria were measured from species living in other temperature conditions. They found that fishes were observed to be resident in lagoons with temperatures of up to 42.8°C, which is higher than the upper temperature limit reported previously. Their results suggested that acclimatisation and acclimation can extend the thermal tolerance as has been reported for numerous other ectotherms (Cossins and Bowler, 1987), the ability of mitochondria to utilise glutamate at high rates may reflect the unusual pattern of nitrogen metabolism in this species. Their results described only modest evolutionary adjustments in the maximal rates of mitochondrial respiration in fish living at different temperatures.

Furthermore, Blier and Guderly (1993) determined the effect of temperature and pH on the sensitivity of mitochondrial respiration *in vitro*. To reproduce the physiological conditions in which mitochondria normally function, they chose pyruvate and malate as substrates and the kinetics of ATP synthesis by mitochondria from rainbow trout red muscle were studied at three temperatures and under two pH regimes. The apparent Michaelis constant (K_m) for ADP decreased with increasing temperature whilst the V_{max} increased . It was suggested that reduced temperature decreased mitochondrial sensitivity to control ADP availability . Apart from the studies of Newell and Pye (1967) already mentioned, where mitochondria were isolated from *Schistocerca gregaria,* less attention has been paid to isolated insect mitochondria. The results of that work can be discounted because insect mitochondria lack the ability to metabolise exogenous succinate, the substrate chosen by Newell and Pye (1967).

In a more substantial study, Davison (1971a) using blowflies showed a marked and complex temperature sensitivity of State III sarcosomal respiration using glycerol 3-phosphate as a substrate. The following experimental work was designed to determine the direct effect of temperature on mitochondrial function using both glycerol-3-phosphate and pyruvate plus proline as substrates.

The experiments were carried out comparing mitochondria from control and thermotolerant blowflies. The data will be related to the comparable data obtained from mitochondria of blowflies heated *in vivo*.

5.2 MATERIALS AND METHODS .

5.2.1 Maintenance of flies:

The blowflies used in this study were male and female 10 day-old adults developmentally acclimatised to 24°C. The rearing methods were the same as described in chapter 2.

5.2.2 Thermotolerant flies rearing:

Batches of 100 10-day-old flies (Durham stock), were subjected to a sublethal thermal shock (36°C or 40 min) and then returned to their culture temperature (24°C) for 4 h to allow maximal development of thermotolerance (see Figure 3.4 in Chapter 3).

5.2.3 Mitochondrial isolation and oxidative phosphorylation:

Mitochondria were isolated from control (not pre-treated) and thermotolerant flies following the methods set out in Chapter 4. Oxidative phosphorylation was determined polarographically using 33 mM rac glycerol-3-phosphate or 2 mM pyruvate plus 2 mM proline, all final concentrations. Measurements of State III were made in the presence of 0.5 μmole ADP and State IV was determined following the esterification of the added ADP . RCI and ADP:O values were determined as described in Chapter 4.

5.2.4 Effect of temperature on isolated mitochondrial preparations:

Oxidative phosphorylation was followed at 5°C intervals in the temperature range 19 to 39°C, the water bath was maintained at the selected temperature ± 0.1°C. This water was circulated around the reaction chamber, maintaining the reaction medium at the selected temperature. The oxygen concentration of the reaction medium was obtained from data presented by Davison (1970). An aliquot of the mitochondrial

suspension was used to determine State III and State IV respiration using Glycerol 3-phosphate. A second aliquot was taken to determine the respiratory rates using pyruvate + proline. The temperature of the water bath was reset for the next temperature and further aliquots of mitochondrial suspension were used to establish mitochondrial State III and State IV respiration at the new temperature with each substrate. Each preparation of mitochondria could be used in this way to follow respiration at not more than three experimental temperatures, because no preparation was used beyond 30 min after isolation. Forty separate preparations were used to obtain the data presented in Table 5.1 and Figure 5.1 and 5.2 In this way the mean data for each experimental temperature was obtained from 10 different mitochondrial preparations.

5.3 RESULTS.

In these experiments the endogenous respiration of mitochondria was not influenced by the experimental temperature and the oxygen consumption values were small, in agreement with the results presented in Chapter 4, Van den Bergh (1962), Davison and Bowler (1971) and therefore these values were not presented.

The data obtained for the effect of *in vitro* temperature on mitochondrial respiration are shown in Table 5.1 and Figures 5.1 and 5.2.

5.3.1 Effect on State III and State IV oxidation:

Non-pretreated control flies:

(i) Control mitochondria when glycerol-3-phosphate is used as substrate:

Table 5.1 shows the mean data for substrate rate oxidation and as can be seen, it was not very temperature dependent. It increased only from a QO_2 of 30.15 ± 2.7 at 19 °C to 38.85 ± 2.71 μg AO mg protein^{-1} h^{-1} at 34°C (Q_{10} = 1.23). At 39°C, however, a sharp increase in QO_2 occurred to 58.91 ± 3.54 μg AO mg protein^{-1} h^{-1} ($P < 0.001$) over the QO_2 at 34°C (Q_{10} = 2.3).

In contrast the mean data for State III respiration was markedly affected by temperature Figure 5.1 and Table 5.1. As compared with the rate at 24°C determination at 19°C sharply reduced the QO_2 from 81.83 ± 2.53 to 47.66 ± 1.79 μg AO mg protein^{-1} h^{-1} ($P < 0.001$) with a Q_{10} of about 3. Increasing the temperature to 29°C, however, had an insignificant effect on the rate and QO_2 equalled 89.74 ± 7.64 μg AO mg protein^{-1} h^{-1}. A further rise in measuring temperature to 34°C caused a marked fall on QO_2 to 51.34 ± 4.86 μg AO mg protein^{-1} h^{-1} ($P < 0.001$). Increasing

the temperature to 39°C did not lead to a further decrease in respiration and a QO_2 of 61.52 ± 3.54 μg AO mg protein^{-1} h^{-1} was obtained.

The mean data for State IV respiration followed the same pattern as described for substrate respiration between 19°C and 29°C, Table 5.1 and Figure 5.2. Respiration increased from a QO_2 of 28.88 ± 2.26 at 19°C to 40.25 ± 1.74 μg AO mg protein^{-1} h^{-1} at 29°C, a Q_{10} of 1.3. However, at 34°C and 39°C State IV respiration could not be established as coupled respiration was not observed. The mean values used are therefore those obtained for respiration in the presence of ADP.

(ii) Control mitochondria when pyruvate + proline were as substrate:

Table 5.1 also shows the mean data obtained using pyruvate plus proline. Substrate respiration was significantly higher when measured at 19°C as compared to 24°C, QO_2 values obtained were 10.15 ± 0.83 and 4.48 ± 0.18 μg AO mg protein^{-1}h^{-1} respectively ($P < 0.001$). Substrate rate respiration rose significantly from a QO_2 of 4.47 ± 0.44 at 29° to 6.23 ± 0.33 μg AO mg protein^{-1}h^{-1} at 39°C ($P < 0.01$).

Figure 5.1 and Table 5.1 show that maximal mean values for State III respiration was obtained at 24°C (QO_2 = 50.10 ± 2.65 μg AO mg protein^{-1}h^{-1}). At 19°C the mean QO_2 was 39.25 ± 4.68 ($P < 0.05$), and the mean also fell significantly to 24.3 ± 1.55 μg AO mg protein^{-1} h^{-1} at 29°C ($P < 0.001$) and progressively to 17.16 ± 1.84 μg AO mg protein^{-1}h^{-1} at 39°C.

The mean data for State IV respiration followed the pattern described for substrate rate respiration with pyruvate plus proline. The highest mean value for QO_2 was obtained at 19°C, 12.25 ± 1.3 which fell to 5.43 ± 0.06 μg AO mg protein^{-1}h^{-1} at 39° C.

5.3.2 Pretreated Thermotolerant flies:

(i) Thermotolerant mitochondria using glycerol-3-phosphate as substrate:

Table 5.1 shows the mean data obtained for substrate rate, which is little affected by temperature between 19 and 29°C, with QO_2 increasing insignificantly from 30.88 ± 2.26 at 19°C to 32.17 ± 1.52 µg AO mg protein^{-1}h^{-1} at 29°C. However, a rise in temperature to 34°C significantly increased mean QO_2 to 42.38 ± 2.52 ($P < 0.001$), which rose again to 55.61 ± 3.13 µg AO mg protein^{-1}h^{-1} at 39°C ($P < 0.001$), a Q_{10} of 1.7 (29-39°C).

Figure 5.1 and Table 5.1 show the effect of measuring temperature on State III respiration. What is interesting about these mean data is their relative temperature independence. The mean QO_2 value obtained at 19°C was 69.13 ± 7.19, and at 24°C it was 67.17 ± 1.83 µg AO mg protein^{-1}h^{-1}. A small rise occurred at 29°C when the mean QO_2 was 76.38 ± 1.41 µg AO mg protein^{-1}h^{-1}, which was not increased further when the temperature rose to 34°C. A Q_{10} of 1.07 was obtained for the mean State III respiration between 19 and 34°C. A further rise in temperature to 39°C, however, caused a fall in mean QO_2 to 59.44 ± 3.31 µg AO mg protein^{-1} h^{-1} ($P < 0.001$).

The mean data for State IV respiration are shown in Figure 5.2. These data follow a very similar pattern as described for substrate rate respiration with a marked temperature independence of QO_2 over the range 19 to 34°C (Q_{10} = 1.2). State IV respiration was not demonstrated at 39°C and oxidative phosphorylation was not coupled.

(ii) Thermotolerant mitochondria when pyruvate plus proline were substrates:

Substrate rate respiration gave the highest mean value at 19°C with a QO_2 of 11.73 ± 3.1 μg AO mg protein^{-1} h^{-1}. The mean value fell progressively to give a low value of 1.83 ± 0.07 μg AO mg protein^{-1} h^{-1} at 29°C. However, between 24 and 39°C substrate rate respiration was relatively temperature insensitive.

State III respiration in mitochondria from thermotolerant flies was also relatively temperature independent, (see Figure 5.1 and Table 5.1) The highest mean value for QO_2 was obtained at 24°C, 28.26 ± 1.6 μg AO mg protein^{-1} h^{-1}, not significantly different for the QO_2 at 19°C. There was a tendency for mean QO_2 to fall with rising measuring temperature to give lowest value at 39°C of 17.18 ± 1.95 μg AO mg protein^{-1} h^{-1}. The Q_{10} over the temperature from 24 to 39°C was 0.72.

Figure 5.2 and Table 5.1 showed that the effect of temperature on mean State IV respiration closely follows that described for the substrate rate. The highest mean value obtained for QO_2 was 10.32 ± 1.43 μg AO mg protein^{-1} h^{-1} at 19°C. This fell to give the lowest mean value for QO_2 of 2.70 ± 0.13 μg AO mg protein^{-1} h^{-1} at 29°C. Higher mean values were obtained at 34°C (5.14 ± 0.31 μg AO mg protein^{-1} h^{-1}) and 39°C (3.16 ± 0.86 μg AO mg protein^{-1} h^{-1}), but overall mean State IV respiration fell with measuring temperature increase with a Q_{10} of 0.55.

5.3.3 The effect of measuring temperature on ADP:O and RCI values:

Respiratory control is widely used as index of the tightness of coupling of oxidative phosphorylation. Values of these parameters are summarised in Table 5.2

Non-pretreated control mitochondria:

(i) With glycerol-3-phosphate as substrate:

The mean value of RCI was not affected by a reduction in experimental temperature from 24°to 19°C. Increasing the temperature above 29°C resulted in reduced mean RCI values, compared with 24°C, so that at 34°C the value was significantly lower ($P < 0.001$) and at 39°C they were not significantly different from 1, (see Table 5.2).The theoretical maximum value for ADP:O, when glycerol-3-phosphate as substrate is 2. As can be seen (Table 5.2) the highest value was obtained at 24°C were ADP:O was found to be 1.92 ± 0.06. ADP:O values were significantly lower at 19° and 29°C than that at 24°C ($P < 0.01$). It is not possible, using polarographic techniques to determine ADP:O when very low values for RCI are obtained, consequently ADP:O values were not demonstrable at higher experimental temperatures, i.e. at 34 and 39°C.

(ii)With pyruvate plus proline as substrates:

Reduction in experimental temperature from 24 to 19°C caused a significant fall in the value of mean RCI ($P < 0.01$), (see Table 5.2). RCI values fell progressively from 5.75 ± 0.13 at 24°C to 4.1 ± 0.54 at 29°C ($P < 0.05$), then to 3.57 ± 0.42 and 3.15 ± 0.4 at 34 and 39°C, respectively ($P < 0.001$, in both cases). Compared with the value at 24°C, ADP:O fell significantly with increasing temperature ($P < 0.01$, in all cases) and but was still 2.18 ± 0.3 at 39°C ($P <0.01$).

Thermotolerant mitochondria:

The effect of temperature on mean values of RCI and ADP:O mitochondria from flies made thermotolerant are also shown in Table 5.2.

(i)With glycerol-3-phosphate as substrate:

Analysis of data from these mitochondria showed that reducing measuring temperature from 24 to 19°C did not affect the mean values obtained for RCI. In contrast to control mitochondria, RCI was still demonstrable (i e. significantly different from 1) at 34°C although it had fallen significantly to 1.69 ± 0.14 from the values of 2.24 obtained at 24 and 29°C ($P < 0.01$). However, at 39°C respiratory control was not demonstrable (RCI = 1). The mean RCI of the thermotolerant mitochondria was significantly higher than that of control mitochondria at 34°C ($P < 0.05$). An increase in experimental temperature caused a reduction in mean ADP:O values below those obtained at 24°C. However, this was only significant at 29°C ($P < 0.01$). An ADP:O of 1.56 ± 0.06 was obtained at 34°C in contrast to control mitochondria where it was not measurable, but ADP:O could not be measured at 39°C in thermotolerant mitochondria either.

(ii) With pyruvate plus proline as a substrate:

Reducing temperature from 24 to 19°C the mean value for RCI was significantly lower ($P < 0.01$) but ADP:O values were unaltered. The highest value for RCI (7.84) was obtained for thermotolerant mitochondria at 29°C, which was significantly higher than the value obtained at 24 ($P < 0.01$), 34 and 39°C ($P < 0.001$ and 0.01 respectively). ADP:O values fell with increasing experimental temperature above 24°C, but were significantly lower only at 34 and 39°C ($P < 0.01$ in both cases). At no temperature did the ADP:O values differ between the control and thermotolerant mitochondria when pyruvate plus proline were substrates.

Table 5.1 The effect of experimental temperature on State III and State IV respiratory rate of mitochondria isolated from flight muscle of control (non-pretreated) and thermotolerant (pre-treated) blowflies. The data are shown as means ± S.E.M for respiration using glycerol 3-phosphate and pyruvate + proline as substrates.

Tests for the significance of differences betweeen the mean values obtained at 24°C and other experimental temperatures were made using Students's *t*-test of Mann-Whitney *U*-test as appropriate;

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Temperature (°C)	QO_2	Non-Pretreated Control		Pretreated Control		N
		glycerol .3-phosphate	pyruvate and proline	glycerol 3-phosphate	pyruvate and proline	
19	substrate rate	30.15±2.70	10.15±0.83	30.28±2.26	11.73±3.1	10
	state III	47.66±1.79***	39.25±4.68*	69.13±7.19	27.41±1.65	10
	state IV	28.88±2.26	12.25±1.30	33.12±1.79	10.32±1.43	10
24	substrate rate	35.95±0.57	4.48±0.18***	26.74±1.22	5.55±0.17	10
	state III	81.83±2.53	50.19±2.65	67.17±1.83	28.26±1.60	10
	state IV	37.45±2.03	8.75±1.09	30.97±1.55	6.62±1.90	10
29	substrate rate	37.92±3.66	4.47±0.44	32.17±1.52	1.83±0.07	10
	state III	89.74±7.64	24.3±1.55***	76.38±1.41	21.21±1.98	10
	state IV	40.25±1.74	5.85±0.42	34.6±1.91	2.70±0.13	10
34	substrate rate	38.85±2.71	5.84±0.35	42.38±2.52***	6.65±0.57	10
	state III	51.34±4.86***	22.34±1.81	76.2±2.85	1.92±1.32	10
	state IV	51.34±4.86	6.79±0.67	44.82±3.27	5.14±0.31	10
39	substrate rate	58.91±3.54***	6.23±0.33**	55.61±3.13***	4.3±0.33	10
	state III	61.52±3.54	17.16±1.84	59.44±3.31***	17.18±1.95	10
	state IV	61.52±3.54	5.43±0.063	59.44±3.31	3.16±0.86	10

Figure 5.1 The effect of experimental temperature on State III respiration rate of mitochondria isolated from flight muscle of control (open symbol) and thermotolerant (filled symbols) blowflies. The data are shown as means ± S.E.M for respiration using glycerol 3-phosphate as a substrate (squares) and for pyruvate + proline (circles) . See Table 5.1 for *N* values.

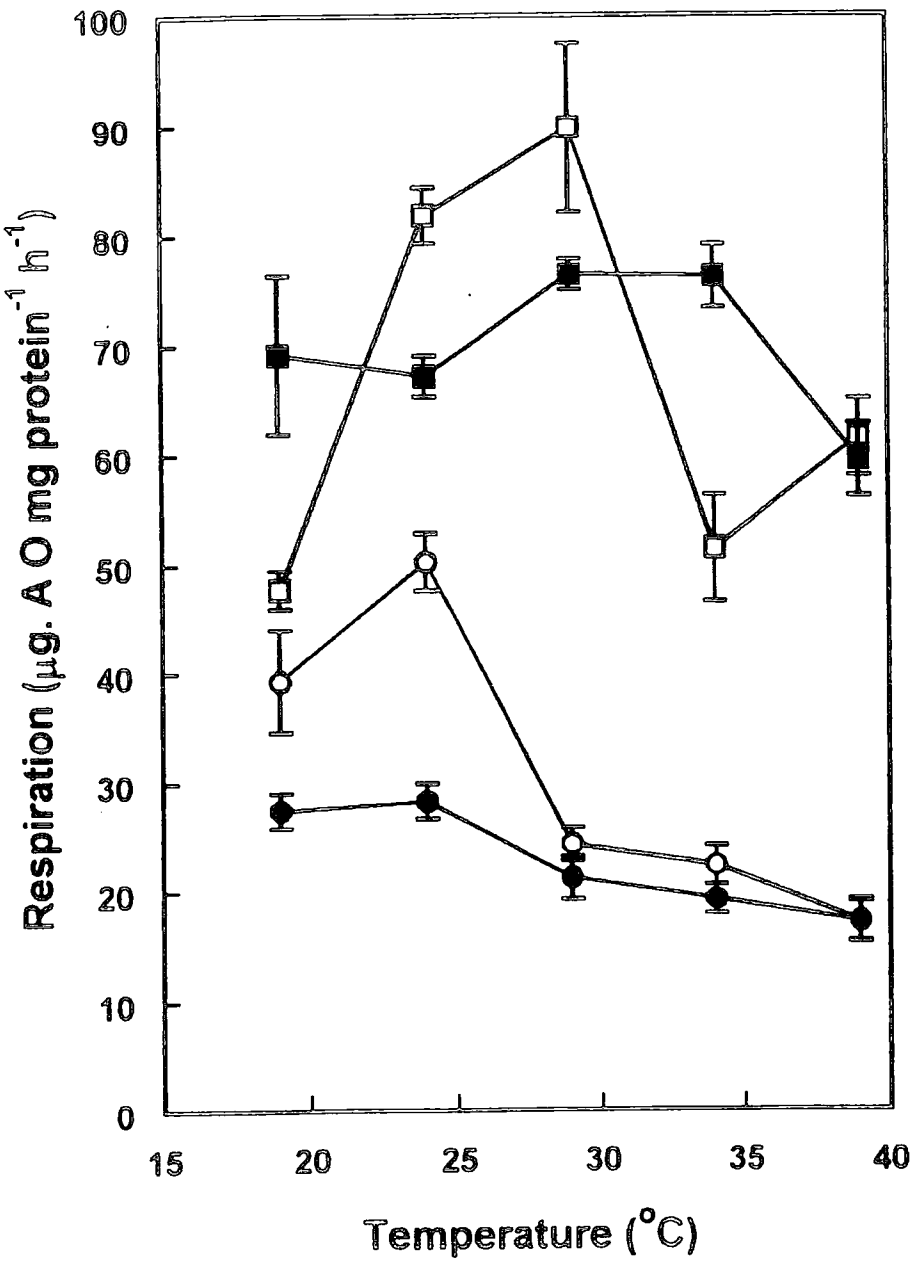

Respiration (µg. A O mg protein-1 h-1)
100
90
80
70
60
50
40
30
20
10
0
15
20
25
30
35
40
Temperature (°C)

Figure 5.2. The effect of temperature on State IV respiration rate of mitochondria isolated from flight muscle of control (open symbols) and thermotolerant (filled symbols) blowflies. The data are shown as means ±S.E.M for respiration using glycerol 3-phosphate as a substrate (squares) and pyruvate + proline (circles).See Table 5.1 for *N* values.

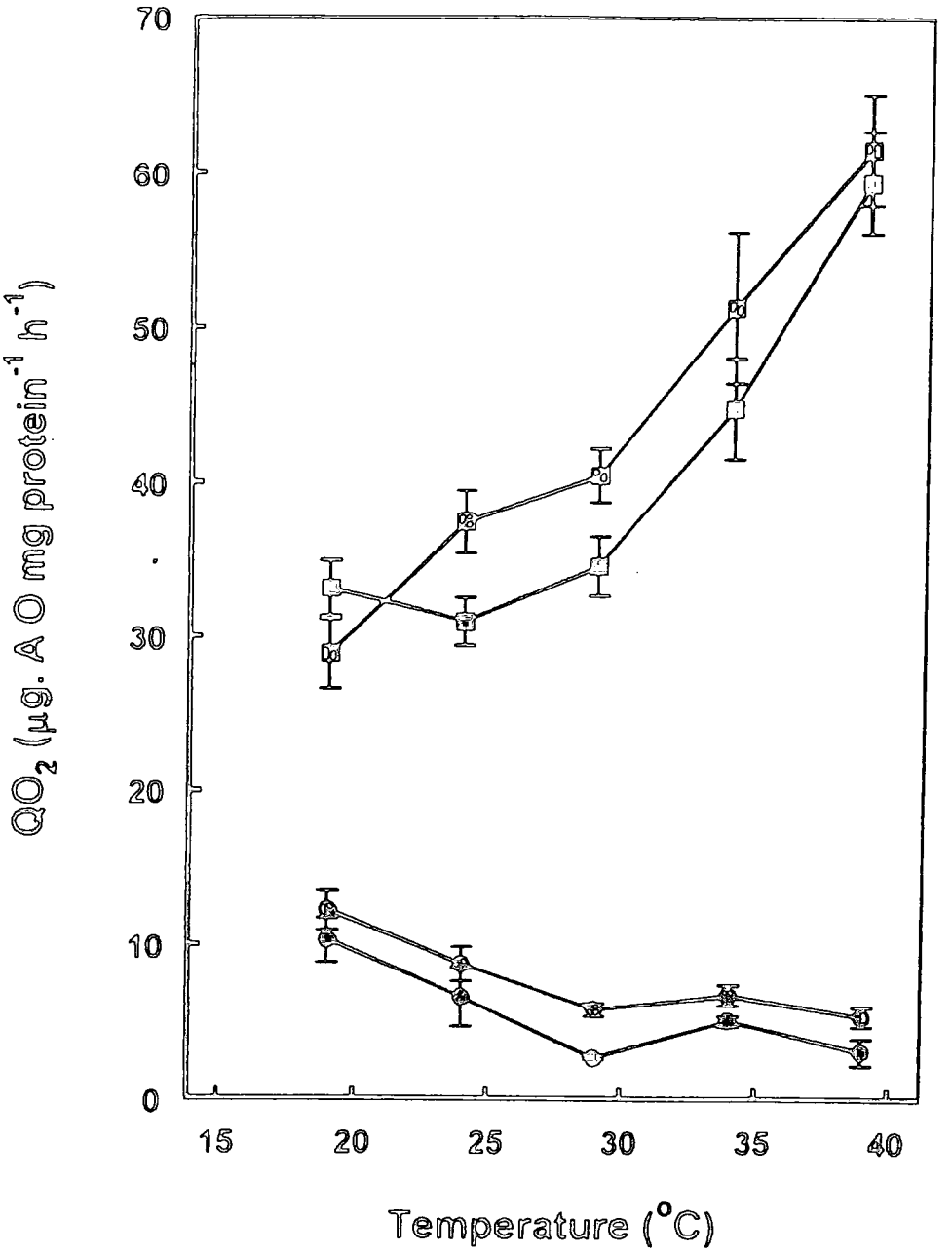

70
60
50
40
30
20
10
0
15
20
25
30
35
40
QO_2 (µg. A O mg protein^{-1} h^{-1})
Temperature (°C)

Table 5.2 The effect of experimental temperature on RCI and ADP:O of mitochondria isolated from flight muscle of control (non pretreated) and thermotolerant (flies given a 40 min pretreatment at 36°C followed by 3h period at 24°C to allow thermotolerance to develop).

Values are means ± S. E. M.

Tests for the significance of differences between the mean values obtained at 24°C and other experimental temperatures were made using Student's *t*-test or Mann-Whitney *U*-test as appropriate;

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Temperature (°C)	Substrate	Treatment	RCI	ADP:O	N
19	G3P	Control	1.80±0.15	1.54±0.03**	10
		TT	2.00±0.12	2.00±0.08	6
	P + P	Control	3.14±0.41**	2.79±0.14	10
		TT	2.66±0.22**	2.49±0.35	6
24	G3P	Control	2.18±0.1	1.92±0.06	20
		TT	2.23±0.07	1.88±0.08	12
	P + P	Control	5.75±0.13	2.94±0.08	20
		TT	4.43±0.5	2.78±0.07	12
29	G3P	Control	2.20±0.11	1.52±0.08	10
		TT	2.24±0.13	1.52±0.08**	10
	P + P	Control	4.10±0.54*	2.53±0.1**	10
		TT	7.84±0.87***	2.60±0.08	10
34	G3P	Control	1.27±0.4***	N.M.	20
		TT	1.69±0.14**	1.56±0.06	20
	P + P	Control	3.57±0.42***	2.18±0.23**	20
		TT	3.70±0.25	2.17±0.14**	20
39	G3P	Control	1	N.M.	15
		TT	1	N.M.	13
	P + P	Control	3.15±0.4***	2.18±0.3**	15
		TT	2.28±0.29***	2.13±0.27**	13

5.4 DISCUSSION

The experimental work carried out in this chapter was planned to address two issues. First, to establish the effect of temperature on respiratory performance of mitochondria *in vitro*, and to relate this to their *in vivo* thermal sensitivity. Secondly, the work considered whether the induction of thermotolerance, which protects mitochondria from *in vivo* heat damage, also protected mitochondria *in vitro.*

The effect of reaction temperature on mitochondrial performance has been followed by many workers. Frequently such studies have been concerned with the determination of oxidation rates, presented as Arrhenius curves, e.g. Lyons and Raison (1970) and Smith (1973c). Relatively few earlier studies, however, have considered the effect of measuring temperature on other mitochondrial functions.

In a comprehensive study on rat liver mitochondria Lee and Gear (1974) studied the rates of mitochondrial membrane-linked reactions as a function of temperature, with the results being expressed as Arrhenius plots. They found that energy dependent reactions involving the adenine nucleotide translocase yielded two distinct discontinuities in their Arrhenius plots, near 17.5°C and 27.5°C. These reactions included ADP phosphorylation, State III and State IV respiration, uncoupled-stimulated respiration, dinitrophenol-stimulated adenosine triphosphatase and ATP-supported calcium uptake. Energy-dependent reactions not involving the adenine nucleotide translocase also gave two breaks in their Arrhenius plots. The upper break was near 27.5°C. However, the temperature at which the lower break occurred was decreased to around 12.5°C. These reactions included respiration-dependent calcium uptake, valinomycin-induced potassium uptake, and phosphate induced swelling, studies with representative matrix, outer membrane and inner membrane enzymes

including ATPase, in sonicated mitochondrial particles indicated that an intact, energy-transducting membrane is required for discontinuities in the Arrhenius plots.

Smith(1973 a, b) also described that the thermostability of some mitochondrial enzymes appeared to be correlated with the environmental temperature range in lower vertebrates, which suggested that natural selection is operating on these enzymes at the cellular level . In a further study (Smith, 1973c), investigated the effect of temperature on integrated mitochondrial processes in which these enzymes participate in the liver mitochondria from cold and warm-blooded animals. He reported the temperature dependence over the range 5 -25°C for State III respiration, as well as succinoxidase and NADH activities, was significantly less for fish than for mammals or avian mitochondria. Arrhenius plots of these rates of oxygen uptake by mammalian mitochondria show an inflection between approximately 12 and 25°C. The actual temperature appeared to vary between different preparations from the same species and with different enzyme systems in the same preparation. Similar plots for fish mitochondria were linear over the temperature range used. Smith (1973) interpreted the lower temperature dependence of fish mitochondria at low temperatures may well be attributed to difference in the constitution and properties of the membranes.

Comparable work on mammalian material has been done by Kemp *et al.*, (1969). They reported that the activation energy of succinate oxidation by rat liver mitochondria changes at a temperature of about 17°C in State III as well as uncoupled respiration. Over the whole temperature range investigated (0-23°C) the rate of phosphorylation of mitochondrial ADP during succinate oxidation exceeded that of added ADP. They also reported that the activation energy of the ADP-ATP and Pi-ATP exchange reactions and of the 2,4 dinitrophenol-induced ATPase also changed at about 17°C. Moreover the temperature coefficients of State III oxidation and of the Pi-

ATP and ADP-ATP exchange reactions are similar and, at temperature below 17°C, are high in comparison with that of phosphorylation of mitochondrial ADP. They therefore, concluded that the translocation of both ADP and ATP through the inner membrane is rate limiting for the process of oxidative phosphorylation in rat-liver mitochondria at all temperatures between 0-23°C.

Christiansen and Kvamme (1969) have reported on the effect of high temperatures on mitochondria *in vitro* from mouse, liver and ascites tumour cells. At the lower temperature used (41°C) they described the first injury as being a decline in respiratory control index without fall in P:O values; at temperatures between 41 and 45°C, however they found that the mitochondria became uncoupled, and phosphorylation was more seriously affected than oxygen consumption. They also reported that, under these conditions, the cytochrome c content of the medium increased, which they interpreted as a result of damage to the mitochondrial membranes. They showed that mitochondria isolated from heat-treated ascites tumour cells were more resistant to heat than were mitochondria heated *in vitro* and they also pointed out that tumour cells showed a greater temperature sensitivity than do normal cells.

The only parallel study to the present one was carried out by Davison (1969) and Bowler and Kashmeery (1981) on *Calliphora erythrocephala* (*vicina*), who presented data for glycerol 3-phosphate respiration that was essentially the same as given in Figure 5.1. Davison (1970) reported a complex effect of temperature on State III respiration. It was inhibited at 19°C as compared with 24°C, and reached a plateau between 24 and 29°C, but respiration was inhibited at 34°C. However, respiration showed a marked increase at 39°C. Davison and Bowler (1971) explained this complex pattern in the following way. The suppression of respiration at 19°C was a Q_{10} effect, but the suppression at 34°C was caused by the impairment of phosphorylation in a

coupled preparation. The subsequent increase in respiration at 39°C resulted from the uncoupling of inhibited phosphorylation from oxidation which then continued via the electron transport chain. The data present in Table 5.1 and Figure 5.1 question that interpretation because the same mitochondrial preparations using pyruvate plus proline as substrates did not show the same pattern of response. With pyruvate plus proline State III respiration was inhibited, as compared with 24°C, at 29°C and all temperatures above. It is significant too that State IV respiration increased with temperature when glycerol-3-phosphate was the substrate, but decreased with pyruvate plus proline as substrate, Figure 5.2.

Considering the differential effect of raised temperature on mitochondrial function using the two substrates together with the inhibitory effect of increased temperature on pyruvate plus proline respiration, it is likely that complex I is sensitive to raised temperature. The reason for the complex pattern of state III respiration using glycerol-3-phosphate is not clear but it may suggest that complex II coenzyme Q cytochrome b functioning is especially temperature sensitive. These questions will be further addressed by the experimental work in chapter 6.

The data in Table 5.2 confirm the earlier work of Davison and Bowler (1971) and Bowler and Kashmeery (1981). With glycerol 3-phosphate as substrate respiratory control (RCI) is lost at 34 and 39°C, in consequence ADP:O ratios were not measurable. In contrast respiratory control is still demonstrable at 39°C when pyruvate plus proline were substrates, although RCI values were significantly lower at 34 and 39 °C ($P < 0.001$ in both cases) than the value at 24°C. Compared with the value at 24°C, ADP:O fell significantly with increasing experimental temperature ($P < 0.01$ in all cases) but was still measurable as 2.28 at 39°C.

The induction of thermotolerance reduced the marked sensitivity to temperature shown for State III respiration with glycerol-3-phosphate. It prevented the suppression of respiration at 19°C and also prevented the sharp reduction seen at 34°C when compared with QO_2 values at 24 and 29°C for State III respiration, so that the rate at 34°C is significantly higher in the thermotolerant group as compared with control group ($P < 0.001$).

With pyruvate plus proline as substrate again the response to measuring temperature is less marked than in control mitochondria. The maximal respiratory rates were obtained with control mitochondria at 24°C, and the rate fell gradually with an increase or decrease in experimental temperature. The induction of thermotolerance also reduced the temperature dependence of state III respiration rates, which gradually fell at temperatures higher than 24°C. In contrast, thermotolerance did not significantly alter the effect of temperature on State IV respiration (Figure 5.2); however, the response with glycerol-3-phosphate and pyruvate plus proline differed. Glycerol-3-phosphate State IV respiration rate increased with rising experimental temperature in a predictable manner (Q_{10}=1.5) whereas with pyruvate plus proline as substrate, it fell. The reason for this differential effect of experimental temperature on state IV with the two substrates is not evident.

Using glycerol-3-phosphate as the substrate, the induction of thermotolerance did not affect either RCI or ADP:O at 24 or 29°C; however, at 34°C, acceptor control could only be demonstrated and ADP:O determined in thermotolerant preparations. With pyruvate plus proline as substrate, the values obtained for RCI differed between control and thermotolerant preparations only at 29°C; no differences in ADP:O were determined between preparations. So the induction of thermotolerance protects mitochondria from subsequent damage and is emphasised by the increased thermal

resistance of both RCI and ADP:O for thermotolerant mitochondria in glycerol-3-phosphate respiration.

The data presented in this Chapter 5 demonstrate that the processes that confer thermotolerance are present not only *in vivo* but are also conserved during isolation and expressed during *in vitro* heating. This suggests that, if heat shock proteins are the protective agents, then they are probably transported into the mitochondria as a result of the pre-treatment shock.

The HSP60 family are the most likely stress proteins involved because they have been shown to be localised in mitochondria (McMullin and Hallberg, 1987). They are present in the matrix and are responsible for the assembly of imported protein complexes into that compartment (Ostermann, Horwich, Neupert and Hartl, 1989). Indeed members of this family of HSPs are reported to be bound to the F_1F_0ATPase of *Heliothus* mitochondria (Miller, 1987). If thermotolerance caused increased levels of HSP60 which binds to a variety of matrix proteins and the F_1F_0ATPase then these may be protected from the inactivating perturbation of heating. What is also possible is that it is this association between mitochondrial proteins and HSPs that modifies the temperature characteristics of the enzymes as is evident in Figure 5.1.

This work also shows that membrane-dependent processes are susceptible to thermal damage and are sensitive in the same temperature range as that which impairs function in the intact organism.

CHAPTER VI

THE EFFECT OF *IN VITRO* HEAT TREATMENTS ON BLOWFLY MITOCHONDRIAL FUNCTION: EFFECTS ON PARTIAL REACTIONS OF THE RESPIRATORY CHAIN

6.1 INTRODUCTION

The data reported in chapters 4 and 5 and show that mitochondrial functions were impaired by *in vivo* heating and also at high temperatures in *vitro*. The temperature sensitive sites however are not evident from these data. It is significant however, that in mitochondria from flies given an LD_{50} heat dose, State III respiration was impaired with both glycerol 3-phosphate and pyruvate as substrates, whereas State IV respiration was inhibited with glycerol 3-phosphate substrate but stimulated with pyruvate plus proline as substrates. This suggested that heating did not generally uncouple oxidative phosphorylation, and that differential sensitivity of parts of the respiratory chain may occur. This could be approached with the use of specific inhibitors of the respiratory chain complexes. Figure 6.1 represents the currently held interpretation of the organisation of the inner mitochondrial membrane of animal mitochondria. The points of entry of the substrates used in these experiments are shown as are the sites of action of the inhibitors, rotenone, antimycin A.

As is shown in Figure 6.1 the electron transport chain (ETC) is organised into four complexes. These are,

Complex I. NADH Coenzyme Q reductase: inhibited by rotenone.

Complex II. Succinate coenzyme Q reductase

Complex III. Coenzyme Q - cytochrome c reductase inhibited by antimycin A

Complex IV. cytochrome oxidase; inhibited by CN^-

Cytochrome c (reduced) + 1/2 O_2 $\rightarrow$ cytochrome (oxidised) + H_2O.

The sequence of the events in electron transport by the inner mitochondrial membrane has been corroborated by the determination of the standard reduction potentials of the redox components of the complexes, and has been used to determine the stoichometry of the ETC coupled ATP synthesis. The standard reduction potential

change along the ETC provides sufficient free energy change to drive the synthesis of one ATP molecule from ADP and inorganic phosphate at complex I, complex III and complex IV. Thus for the flow of electrons from NADH to O_2 three molecules of ATP can be generated, whereas from glycerol 3-phosphate only two would be generated.

The coupling between ATP synthesis and the flow of electrons to O_2 is obligatory in intact mitochondria. Therefore, if no ADP is present to enable ATP synthesis then electron transfer is also reduced (State IV respiration). Associated with the flow of electrons to O_2 is the extrusion of H^+ which creates a proton gradient across the inner membrane. The inner mitochondrial membrane is impermeable to protons and it is thought that proton re-entry across this membrane occurs via the F_1F_0 ATPase. This is proposed to be the energy conserving process that drives ATP synthesis (Mitchell, 1979). In consequence compounds (uncouplers) that discharge the proton gradient (e.g. DNP and FCCP) will inhibit phosphorylation but permit oxidation to continue unhindered by the requirement for ADP. It is usual that oxidation rates of uncoupled mitochondria are at least as high as those in the coupled State III respiration.

Relatively few studies have attempted to locate the most sensitive site on oxidative phosphorylation to thermal perturbation. Morris and King (1962) using a heart muscle preparation described that inhibitors-sensitive activities for various steps in the oxidation of DNPH (NADH) had remarkably different thermal sensitivities, with cytochrome oxidase being relatively thermostable whereas the step between DNPH flavoprotein and cytochrome c was the most sensitive. Christiansen and Kvamme (1969) in a similar study considered the effect of temperatures up to 45°C *in vitro* on mitochondria from mouse brain and liver and from Ehrlich ascites tumour cells. These workers also found that electron transport between succinate to cytochrome c was more sensitive to temperature than that between cytochrome c and oxygen. They

concluded that loss of respiratory control and uncoupling of phosphorylation were likely to be the primary effects of heat treatment. More recently Pobezhimova, Vonikov and Varakina (1996) studied the effects of elevated temperatures on the function of mitochondria from *Zea mays*. These workers also found that complexes II, III and IV were thermostable, but complex I was found to be very sensitive, inhibition of this complex accounted for the inhibition recorded when the whole chain was studied.

The aim of the present Chapter is to determine where the principal site for inhibition of blow fly mitochondrial function following *in vivo* heating, and whether the development of thermotolerance protects this site from heat damage.

6.2. MATERIAL AND METHODS:

6.2.1 Experimental blowflies:

Flies were reared as stated in Chapter 2. The procedures used to give an *in vivo* LD_{50} treatment, and to develop thermotolerance were described in Chapter 4

6.2.2 Measurement of oxidative phosphorylation:

Oxidative phosphorylation was measured polarographically at 24°C using a Clark electrode as described in Chapter 4. Oxidation in coupled mitochondria was initiated by the addition of pyruvate (2 mM) and proline (2 mM), and State III respiration was restarted by the addition 0.5 μmole ADP. After State IV respiration was established, on the esterification of the added ADP, the mitochondria were uncoupled by the addition of 0.4 mM Carbonylcyanide p-trifluoromethoxyphenyl hydrazone (FCCP). Uncoupled respiration was followed to allow determination of its rate before rotenone was added to inhibit activity of complex I. Respiration was then restarted by the addition of 33 mM glycerol 3-phosphate and was followed to establish its rate, after which antimycin was added to inhibit the activity of complex III. Respiration was then restarted by the addition of 100 mM potassium ferrocyanide, which will donate electrons to cytochrome c, and respiration associated with complex IV was then followed. The data are shown in Figure 6.2 and Table 6.1.

6.2.3 Mitochondrial protein determination:

This was carried out as described in Chapter 4.

Reagents:

Chemical used in this study were purchased from Sigma chemical Co., Ltd.

Statistical tests:

The means ± S.E.M. for all respiratory rates compared using Student's *t*-test .

6.3. RESULTS:

Figure 6.2 shows a typical polarographic trace of oxidative phosphorylation in the presence of the various substrates and inhibitors. As can be seen control mitochondria from (untreated flies) showed normal coupled behaviour following their isolation. Values obtained for respiratory control indices (6.37 ± 1.14) and ADP:O were similar to those shown earlier in Table 4.1, as were the values obtained for State III and State IV respiration. On the addition of FCCP State IV respiration increased from 7.31 ± 0.8μg A O mg. protein^{-1} h^{-1} to 44.91 ± 3 μg A O mg. protein^{-1} h^{-1} a value not significantly different from the State III level obtained (46.56 ± 2.8μg A O mg protein^{-1} h^{-1} It is notable that respiration initiated by the addition of glycerol 3-phosphate (44.36 ± 3.2 μg A O protein^{-1} h^{-1}) also did not differ from State III respiration, whereas respiration supported by ferrocyanide was significantly lower at 34.25 ± 2.4 μg A O mg. protein^{-1} h^{-1} ($P < 0.01$).

As was expected from the data presented in Chapter 4, State III respiration from mitochondria isolated from LD_{50} treated flies was markedly reduced to 25.47 ± 2.06 μ g A O mg protein^{-1} h^{-1} from that obtained in control mitochondria ($P < 0.001$), which together with a non-significant rise is State IV respiration caused a fall in the value of RCI to 2.62 ± 1.92 ($P < 0.05$) ADP:O values were also low in accordance with the data presented in Chapter 4. Uncoupling with FCCP stimulated respiration from State IV levels to 23.85 ± 3.11 μg A O mg. protein^{-1} h^{-1}, a level not significantly different from that obtained for State III in these mitochondria. It is interesting that the addition of G 3P increased oxidation rates to 37.74 ± 3.8 μg A O mg. protein^{-1} h^{-1}, significantly higher than State III respiration in these mitochondria before uncoupling with FCCP ($P < 0.01$), and not different from state III respiration of control mitochondria. Antimycin

A inhibited this high rate of oxidation was inhibited. Potassium ferrocyanide stimulated respiration was 26.05 ± 2.2 μg A O mg. protein^{-1} h^{-1}, a value lower that found using G3P as substrate (and also was lower than that obtained from control mitochondria ($P < 0.05$).

Data obtained from LD_{50} treated thermotolerant flies is also given in Table 6.1. As can be seen State III respiration is reduced as compared with that for control mitochondria ($P < 0.05$), but is also higher than that obtained for mitochondria from LD_{50} treated control flies ($P < 0.01$). These data, together with the higher values obtained for RCI, confirm that thermotolerance protects mitochondrial function from heat damage. Uncoupling of State IV respiration with FCCP restored oxidation rates to State III levels (39.23 ± 3.2 μg A O mg. protein^{-1} h^{-1}), which were not different from those obtained in uncoupled control but were significantly higher than those from LD_{50} treated mitochondria ($P < 0.01$) Rotenone inhibited this uncoupled respiration, and G 3P supported respiration rates (38.73 ± μg A O mg. protein^{-1} h^{-1}) that were not different from either State III or FCCP uncoupled respiration. Antimycin A inhibited G 3P respiration was stimulated by the addition of ferrocyanide which was restored to the same level as that found for State III respiration, FCCP uncoupled and G 3P supported respiration, was significantly higher than that for LD_{50} treated mitochondria ($P < 0.05$).

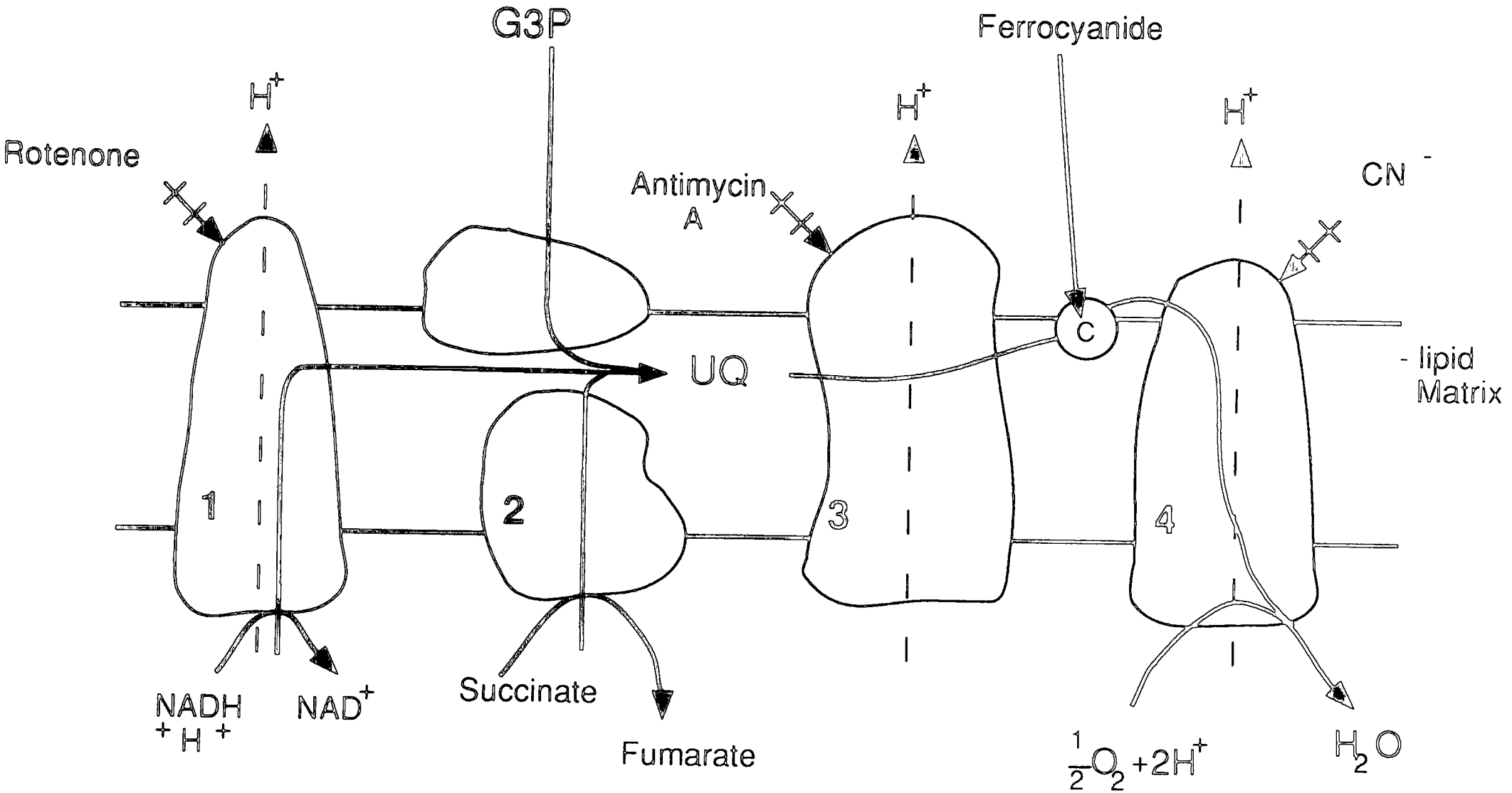

G3P
Ferrocyanide
H+
H+
H+
Rotenone
Antimycin
A
CN -
UQ
C
- lipid
Matrix
1
2
3
4
NADH
+ H +
NAD+
Succinate
Fumarate
½O2 +2H+
H2O

Figure 6.1. Schematic representation of organisation of the respiratory complexes that make up the electron transport chain in blowfly flight muscle mitochondria. The points of entry of substrates are shown as are the targets of the inhibitors used.

Figure 6.2. A representative polarographic traces of mitochondria respiration. Effect of respiratory chain inhibitors and different substrates.

Respiration was initiated with 25µl pyruvate + proline. Oxidative phosphorylation was demonstrated with the addition of 0.5µmole ADP. Mitochondria were uncoupled by the addition of FCCP.

Arrows indicate additions of pyruvate + proline (p + p), mitochondria (S), 0.5µmole ADP (ADP), uncoupler FCCP (U), rotenone (R), glycerol-3-phosphate (G3P), antimycin A (A), and K. ferrocyanide (F).

Trace 1 shows results from mitochondria isolated from control flies.

Trace 2 shows results from mitochondria isolated from thermotolerant flies.

Trace 3 shows results from mitochondria isolated from LD_{50} treated flies.

Complex I was inactivated with rotenone.

Complex III was inactivated with antimycin A.

Respiration through complex II was initiated with 33 mM G 3P; through complex IV.

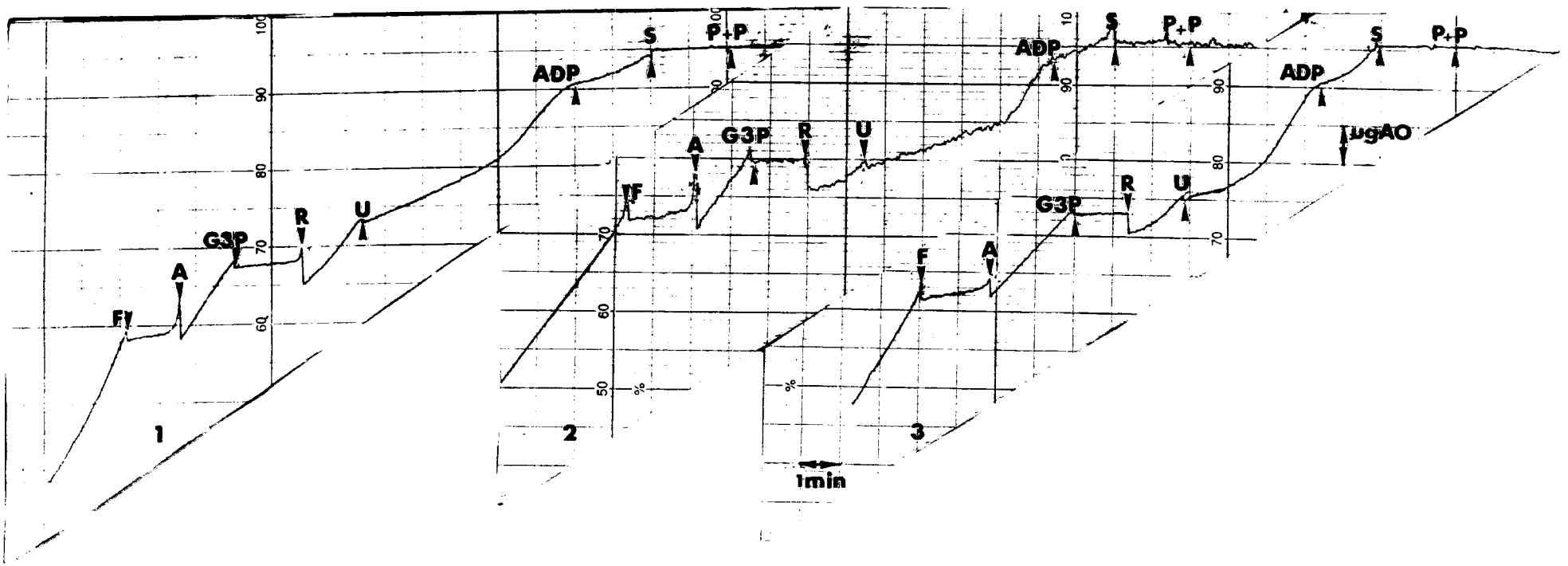

F
A
G3P
R
U
ADP
S
P+P
1
2
3
%
1min
1µgAO

Table 6.1. The effect of in vivo heat treatment on respiratory function in blowfly flight muscle mitochondria: Effect on the respiration chain in mitochondria from control, (non-pretreated), LD_{50} treated control and LD_{50} thermotolerant flies.

Respiratory rates expressed in μg AO mg protein^{-1} h^{-1}, means ± S.E.M.

The mean values for LD_{50} control and LD_{50} thermotolerant groups were compared with those from control mitochondria. Statistical differences were derived using Student's *t*-test; $^{*}P < 0.05$; $^{***}P < 0.001$

Treatment	Respiratory rate											N
	State III	State IV	ADP	RCl	FCCP	Rotenone	RCl	G 3P	Antimycin A	RCl	K-ferro-cyanide	
control non-preheated	46.56 ± 2.8	7.31 ± 0.80	2.60 ± 0.08	6.37 ± 1.14	44.91 ± 3.00	10.11 ± 0.92	4.4 ± 1.10	44.36 ± 3.2	14.77 ± 3.2	3.00 ± 1.8	34.25 ± 2.4	10
LD_{50} Thermotolerant	37.63 ± 2.5*	8.17 ± 0.92	2.49 ± 0.11	4.61 ± 1.00	39.23 ± 3.20	9.12 ± 0.62	4.3 ± 1.00	38.73 ± 4.00	12.20 ± 1.11	3.17 ± 2.03	37.40 ± 2.60	10
LD_{50} treated	25.47 ± 2.06***	9.72 ± 0.83	2.18 ± 0.06	2.62 ± 1.02*	23.85 ± 3.11***	8.20 ± 0.70	2.91 ± 1.12	37.74 ± 3.8*	10.72 ± 1.10	3.52 ± 2.17	26.05 ± 2.2*	10

6.4. DISCUSSION:

In Chapter 4 it was shown that *in vivo* heating impaired oxidation of substrates by isolated mitochondria that was accompanied by a sharp reduction in RCI. The lower values for RCI mainly resulted from a reduction in State III respiration because there was no marked rise in State IV respiration. This suggested that there was no significant loss in the integrity of the mitochondrial membrane (Chance and Williams, 1956). The reduction observed could result either from an inhibition of the respiratory chain or from an inhibition of phosphorylation, at one or more sites, with coupling remaining intact.

In earlier studies on mitochondria from mammalian tissues Christiansen and Kvamme (1969) and Morris and King (1962) reported that the site most sensitive to elevated temperature was between the NADH flavoprotein and cytochrome c. The former workers described cytochrome b-complex III as the likely temperature-sensitive site, because succinate oxidation by complex II was not inhibited by high temperature. The present studies confirmed work on blowfly mitochondria which demonstrated that, although G 3P respiration is inhibited in heated mitochondria, when measured using the respiratory chain enzymes, G 3P dehydrogenase (site II) was not inhibited when measured directly using a tetrazolium salt as the electron acceptor, (Bowler and Kashmeery, 1981). Both of the early studies quoted above, using mammalian mitochondria, also found the complex IV was relatively thermally stable. In a more recent study using *Zea mays* mitochondria Pobezhimova *et al.*, (1996) also found that complex II and IV were comparatively thermostable. In contrast to earlier studies these workers also found that complex III was stable but complex I of the respiratory chain was very sensitive to elevated temperature.

The work presented in this Chapter differs from the earlier studies discussed above because mitochondria were exposed to high temperature *in vivo*, whereas the earlier studies concerned the effect of *in vitro* heating. Nevertheless, it is clear that *in vivo* heat dose (LD_{50}) significantly impaired mitochondrial function as respiration of NAD-dependent substrates is reduced by almost 50% (Table 6.1). That this owing to inhibition of the respiratory chain, rather than phosphorylation is witnessed by failure to stimulate State IV respiration to the same level as State III in controls by uncoupling oxidative phosphorylation with FCCP.

Rotenone inhibited the activity of FCCP stimulated respiration in both control and LD_{50} treated flies to the same level. This respiration was stimulated by G 3P, and although that from LD_{50} treated mitochondria was lower than that of control mitochondria, the difference was not statistically higher than FCCP stimulated respiration with NAD-dependent substrates. This indicates that oxidation of substrates through complex I is a major site of damage to mitochondrial function in LD_{50} treated flies. A further point of significance is to compare G 3P supported respiration in LD_{50} treated mitochondria without, and in the presence of FCCP. In the former case (see Table 4.1 Chapter 4) mitochondrial respiration was substantially reduced (to 27%) as compared with State III respiration of controls, furthermore, State IV respiration was also reduced (to 60%) implying that the mitochondria were not uncoupled by LD_{50} treatment (Bowler and Kashmeery, 1981). However, in the latter case (Table 6.1) respiration was not impaired, thus agreeing with the earlier study which showed that G 3P dehydrogenase was not inhibited by heat (Bowler and Kashmeery, 1981). This suggests that following *in vivo* heating the functioning of Complex III is also affected. As respiration in the presence of an uncoupler is not affected through complex III,

whereas respiration in the absence of FCCP is inhibited, suggests that in mitochondria from LD_{50} treated flies oxidative phosphorylation remains coupled but phosphorylation is heat sensitive. Antimycin A inhibited respiration through complex III, but respiration was restored by ferrocyanide. In control mitochondria this was significantly lower that supported by G 3P. In LD_{50} treated mitochondria this was further reduced, which suggests that complex IV may also be a site of damage in *in vivo* heating.

As can be seen from Table 6.1 making the flies thermotolerant before exposure to an LD_{50} heat dose protects the sensitive sites to heat damage. Although State III respiration of NAD dependent substrates was significantly lower than that of control flies it was significantly higher than from control mitochondria that experienced and LD_{50} dose. FCCP uncoupled mitochondria had respiratory rates not different from control unheated mitochondria, in contrast to mitochondria from LD_{50} treated control flies. This respiration was inhibited by rotenone to the same levels in the three groups. Glycerol 3-phosphate restored respiration to the same level as found for State III respiration, and was not significantly different from G 3P respiration in the control or LD_{50} treated control mitochondria. As the development of thermotolerance also protected mitochondria from the damaging effects of LD_{50} heating (see Table 4.1) with Glycerol 3-phosphate as substrate, then it follows that phosphorylation at complex III must also be protected. Antimycin A inhibits respiration through complex III to the same level for mitochondria from the three treatments. Ferrocyanide restored respiration in these mitochondria to initial State III levels and the same level as did glycerol 3-phosphate. Thus the suggested damage that occurred at complex IV in LD_{50} treated control mitochondrion was protected by the development of thermotolerance, because

respiration through this complex is significantly higher after LD_{50} treatment in thermotolerant as compared with mitochondria from control flies.

CHAPTER VII

GENERAL DISCUSSION

The work presented in this thesis concerns the study of the effect of high lethal temperatures on the blowfly *Calliphora vicina.* Correlation of the effects of high temperature on the whole organism was sought at the subcellular level. The data collected add to the earlier studies from this laboratory (Davison and Bowler, 1971; Bowler and Kashmeery, 1981) on this problem. The present results are in good agreement with that earlier work on the Durham culture of blowflies. One interesting point of difference however, was that the LD_{50} for 10 day-old adults was significantly lower, at 38.16 ± 0.47°C, than that reported in the earlier studies (40.9 ± 0.10°C), and also from that of a different culture more recently obtained from the University of Cambridge (39.47 ± 0.18°C). The protocol and rearing techniques used have been applied consistently and so it is not known what has caused the marked 1.5°C reduction in the death point observed. The most likely reason is that over the 30 year period of these studies there has been an inadvertent selection of stock with a lower thermal tolerance. The long period over which this research has been carried has brought this issue to notice and it emphasises the care that should be taken when similar research is being carried out with insects in long-term culture; reliance on existing data should not be assumed.

A central part of this study was concerned with the phenomenon of thermotolerance, and whether its induction protected against previously identified, heat-induced, lesions in mitochondrial function, (Davison and Bowler, 1971; Bowler

and Kashmeery. 1981). At the level of the whole organism, we have demonstrated that subjecting flies to a sublethal heat-dose, afforded significant protection to a subsequent LD_{50} heat dose. In common with other studies on insects (Stephanou *et al.,* 1983; Whyard *et al.,* 1986; Yokum and Denlinger, 1992), thermotolerance developed quickly, it was demonstrable within 1 hr of heat shock, but also was of relatively short duration, lasting less than 6 hr. Under the conditions used for heat shock thermotolerance developed maximally after 3-4 hr and produced a 30% increase in survival. As has been pointed out by Hutchison and Maness (1979), and more recently by Rutledge *et al.*, (1987), the phenomenon of thermotolerance (or heat hardening) is of ecological significance, particularly in species that may be subjected to periodic high field temperatures (Whyard *et al.*, 1986). The kinetics of this phenomenon in blowflies, its rapid induction and short duration, are therefore appropriate ecologically. It is of interest too that HSP production occurs in a temperature range that relates to the range tolerated. For example, *Drosophila melanogaster* HSPs are produced by exposure to 37°C and 40°C is usually lethal, whereas in *Manduca sexta* HSP synthesis is maximal at 42°C but lethality is not reached until about 46°C. Thermotolerance is clearly a survival strategy distinct from acclimation and acclimatisation, which are responses to a change in environmental conditions within viable limits. Their time course is in order of days, not hours, and the phenotypic response lasts only as long as do the changed environmental conditions that evoked that response (Cossins and Bowler, 1986; Prosser, 1991).

A major thrust of this study was to understand better the nature of the lesions involved in heat death. The background to the work clearly owed much to the earlier work of Davison 1971; and Kashmeery, 1980. It is significant that the former workers demonstrated that a close correlation occurred between an LD_{50} heat dose *in vivo* and

impairment flight muscle mitochondrial function when measured at 24°C. It was also reported that the flight recovery from such a dose was paralleled by the restoration of normal mitochondrial performance. The recovery from an LD_{50} dose took about 2-3 days at 24°C, when the normal capacity for flight had been regained and the isolated mitochondria had QO_2 values, and RCI and ADP:O ratios similar to those of mitochondria from unheated control flies. The close relationship between organism response to *in vivo* heat exposure and impairment of mitochondrial function, and the observed morphological damage, (Davison, 1971b) suggest that this system provides a good model from the study of cellular heat injury.

The results of the present study confirm and extend the data presented by Davison and Bowler (1971) and Bowler and Kashmeery (1981). An LD_{50} dose caused the impairment of mitochondrial function as witnessed by the reduction RCI and a lowering of the ADP:O ratios. In agreement with the earlier work oxidative phosphorylation with G 3P appeared more severely affected by an LD_{50} than with pyruvate + proline as substrate. In particular, respiratory control was lost and ADP:O was not demonstrable with the former substrate, and State III and State IV respiration were markedly inhibited. With pyruvate + proline as substrate State III respiration was also sharply reduced, but State IV respiration was increased implying that, in this case, some uncoupling may have resulted. Respiratory control was reduced but ADP:O was still measurable. This apparent differential effect of *in vivo* heat on mitochondrial function, with different substrates, was difficult to explain. Kashmeery and Bowler (1981) considered that it implied that heat inactivated phosphorylation, rather than the enzymes of the respiratory chain, in case of G 3P, because there was no evidence of uncoupling of oxidative phosphorylation (i.e. no increase in State IV). The present results using specific inhibitors of the respiratory chain show that the interpretation was

too simplistic. Uncoupling of oxidative phosphorylation of mitochondria from LD_{50} treated flies with FCCP stimulated State IV respiration to State III levels, which still remained below those of control mitochondria. This suggested that oxidation was impaired by *in vivo* heating with mitochondria remaining reasonably well coupled. Furthermore, the restoration of respiration with the addition of G 3P, did restore oxidation rates to the same level as in control unheated mitochondria, a rate that is significantly higher than those obtained on uncoupling with FCCP. This confirms that oxidation at Complex I is sensitive to heat, but respiration through Complex III is not heat inactivated, also confirming that G 3P dehydrogenase is not heat sensitive (Bowler and Kashmeery, 1981). However, when G 3P stimulated respiration is considered in LD_{50} treated mitochondria, without the use of FCCP, then respiration is markedly reduced as compared with controls (see Table 4.1). This could be interpreted to suggest that heating inactivated the phosphorylation process with the mitochondria remaining coupled at Complex III. There is also evidence that respiration through Complex IV is heat sensitive as K ferrocyanide stimulated respiration in LD_{50} treated mitochondria is lower than that of controls.

Only the studies of Floridini *et al* (1987) report work on the effect of *in vivo* heating on mitochondrial function. These workers have also reported that a general inhibition of State III and IV respiration occurred in Ehrlich's ascites mitochondria following hyperthermic treatment of the cells. They interpreted this as a general inhibition of the respiratory chain rather than an effect on phosphorylation as RCI values remained the same as for controls. Other workers have also shown that *in vitro* exposure at high temperatures impaired mitochondrial function, again with oxidation at Complex I being reported as particularly sensitive (Morris and King, 1962; Christiansen and Kvamme, 1969; Pobezhimova *et al.*, 1996). It is also significant that

G3P stimulated respiration was again more sensitive to elevated temperature than pyruvate + proline respiration. For example, Table 5.1 showed that coupling of oxidative phosphorylation was lost at 34°C with the former substrate, but could still be demonstrated at 39°C with pyruvate + proline, albeit with reduced values for RCI and ADP:O as compared with 24°C. The present studies show that mitochondria are much more sensitive to temperatures *in vitro* than *in vivo*, Davison (1969) cultured blowflies at 34°C, but this measuring temperature impair G 3P respiration *in vitro*.

The present work clearly demonstrated that the induction of thermotolerance protects mitochondria from heat damage both *in vivo* and *in vitro*. No other detailed comparable study exists. Mitochondrial function was largely retained following an LD_{50} dose to thermotolerant flies as compared with mitochondria from control flies. However, damage was still evident, G-3-P and pyruvate + proline State III respiration were lower than in control, unheated mitochondria, but coupling was retained with the former substrate, and improved with the latter. Significantly too, the induction of thermotolerance protected mitochondria *in vitro* at higher measuring temperatures, particularly with G-3-P respiration. Table 5.1 clearly shows that coupling was demonstrable at 34°C, in contrast with control mitochondria. One of the most interesting outcomes of this study was that thermotolerance affected the thermal sensitivity of mitochondria over the whole of the temperature range measured. The Q_{10} for the effect of G-3-P State III respiration was about 1.1 (19-29°C) for thermotolerant mitochondria as compared with a value of about 1.9 for control mitochondria over the same temperature range. A similar, if less dramatic effect was seen on pyruvate + proline State III respiration. A comparable effect was not seen on State IV respiration.

Table 6.1 clearly shows that the induction of thermotolerance protects mitochondria from heat damage at Complex I, both State III and FCCP uncoupled respiration are significantly higher in the thermotolerant group as compared with the control LD_{50} treated group. In no case was the respiration rate different between the unheated control mitochondria and LD_{50} treated thermotolerant mitochondria. It is clear from these two sets of experiments that thermotolerance caused changes in the mitochondria that are protective *in vivo* and are retained during isolation so that their protective effects are evident *in vitro*.

This research provides evidence that mitochondrial function is impaired during an in vivo heat dose. The question that arises is whether this can be correlated with cellular and organism heat death (Read, 1967). The loss of flight performance following heat exposure has been previously reported (Davison, 1971a), this showed that the observed impairment of mitochondrial performance is translated into a loss of function. It is likely that a significant reduction in ATP concentration occurred in heat damaged flight muscle, consequent on the observed reduction in respiratory control. Sacktor (1958) and Van den Berg (1962) report that G-3-P is a major substrate in this tissue, and as respiration with this substrate in heat damaged tissue is particularly affected, might account for the loss in flight ability. There are a number of studies that support this interpretation. Lunec and Cresswell (1983) have reported that heating L5178YS cells resulted in a rapid reduction in ATP levels, a result confirmed by Jaing *et al.*, (1991) from ^{31}P NMR studies during heating of human carcinoma cells. Changes in ATP levels have not been determined in the present study, but clearly it would aid the interpretation to confirm that the observed mitochondrial damage is expressed by a reduction in ATP levels. However, it should be stressed that the impairment of mitochondrial function reported is unlikely to be the only lesion suffered by the

blowflies subjected to an LD_{50} heat dose. It would be reasonable to suggest, at least, that mitochondria from other tissues may well be similarly damaged.

The rapid cellular modifications that must occur during the development of thermotolerance have been the subject of considerable investigation. The first reports specific effects heat shock were reported by Ritossa (1962) on chromosome puffing patterns in *Drosophila*. This work led to identification of the synthesis of a specific set of proteins (heat shock proteins) by Tissiers *et al* (1974) induced by the heat exposure. Since that early work several families of heat shock proteins have been identified and characterised on the basis of molecular size, the HSP90, HSP70, and HSP20's families. Even within one organism there may be several molecular forms of each of those families, with a potential for different subcellular localisation, Becker and Craig, (1994). Many of these proteins have a primary 'housekeeping' function, serving as 'molecular chaperones', promoting folding of proteins and the refolding of unfolded proteins, they are also known to be responsible for the translocation of proteins between intracellular compartments (Shi and Thomas, 1992). Another critical aspect of their function, a function that directly relates to the present study, is that HSP60 and 70 have been shown to prevent the aggregation of proteins (Pelham, 1984) and, in the case of HSP100, can resolubilise aggregated proteins once formed (Schirmer, Glover, Singer and Lindquist, 1991). There is considerable information that the application of stresses to cells causes protein unfolding and denaturation.

A strong correlation is reported between the induction of HSPs and the development of thermotolerance (Landry *et al.*, 1982; Li and Laszlo, 1985). Members of all the HSP families have been implicated in the development of thermotolerance (Hightower, 1991; Sanchez and Lindquist, 1990). The protective function afforded by HSPs in heat tolerance is considered to be that they bind to hydrophobic surfaces

exposed by heat denaturation, which allows the opportunity for conservation of function during the stress, and refolding and restoration of function after stress (Becker and Craig, 1994). It is therefore not surprising that cell mutants in specific HSPs are reported to be intolerant of heat stress compared with wild types (Schirmer, *et al.*, 1996), and mutants over expressing HSPs have increased heat tolerance. Not all studies support these proposals that HSPs are responsible for the development of thermotolerance, there have been reports of thermotolerance developing in the absence of elevated synthesis of HSPs and several states of thermotolerance some independent of HSPs have been postulated by Laszlo (1988); Carper et al. (1987); Tomosovic and Koval (1985); Hall (1983).

There are many reports of the production of HSPs in insects as a result of heat shock. In addition to the ubiquitous HSP70 family, most insects also produce HSP80s (Martin, Blaker and Tanguay. 1994), but the presence of small molecular weight HSPs has not always been reported (Fittinghoff and Riddinford, 1990; Lindquist, 1980). The data presented in the present study, on the effects of thermotolerance on mitochondrial function in heat stress, are probably best explained in terms of the production of HSPs. Evidence has been presented in Chapter 4 and 5, that an LD_{50} heat dose impaired the functioning of the complexes of the respiratory chain, and presumably some of their critical proteins are being structurally perturbed. The development of thermotolerance protects these structures from the heat damage, presumably because the HSPs produced are binding to the damaged sites. The evidence, presented in Figure 5.1 Chapter 5 showing a marked temperature independence of State III respiration in thermotolerant preparations as compared with controls, could also be explained as an effect of the binding of HSPs to the respiratory enzymes. This would be likely to

reduce the conformational freedom of the respiratory enzymes so making them less responsive to a change in measuring temperature. Clearly an important development of this research would be to demonstrate that HSP production was increased as a result of the heat shock, and that HSPs were also present in mitochondria isolated from heat shocked flies. The identification of specific HSPs associated with particular mitochondrial proteins would further strengthen this interpretation.

The present work shows that membrane-dependent processes are susceptible to thermal damage in the same temperature range that kills the intact animal, thus supporting the proposal that the primary site of heat damage lies at the site of cellular membranes (Bowler *et al.*, 1973; Bowler, 1987; Manning and Bowler, 1994). There are an increasing number of reports that identify membrane proteins as the site of heat damage (Bowler, 1987; Yatvin and Cramp, 1994; Cheng *et al.*, 1987), but doubt remains as to whether there is a role for the lipid moiety in modulating the damage caused by high temperature. (Konings, 1988; Lepock *et al.*, 1981). In particular the latter paper raised doubt because of a failure to demonstrate an effect of thermotolerance on membrane fluidity. This argument has been extended to the premise that if cellular susceptibility to hyperthermic treatment is causally related to membrane order (fluidity) then there should be a direct relationship between cell survival and fluidity. No consistent evidence exists that this is the case. However, it is of interest that although Dynlacht and Fox (1992) report no relationship between survival and membrane fluidity in a variety of cell lines, hyperthermia caused persistent changes in fluidity when measured post-heating. Furthermore, the more resistant the cell line was to heat injury, the more resistant it was to the perturbation of the membrane fluidity change caused by hyperthermia. The reported persistence of the fluidity change resulting from hyperthermic treatment is most likely to reflect a

denaturational change in membrane proteins (Lepock *et al.,* 1988). A role for lipid membrane order (fluidity) in the modulation of the heat denaturation of membrane proteins has been demonstrated using the catalytic hydrogenation of membrane proteins has been demonstrated using the catalytic hydrogenation of unsaturated fatty acids to produce a more saturated, and less fluid membrane matrix. Quinn and co-workers have described that hydrogenated chloroplast thylakoid membranes showed a reduced tendency for structural disruption of membrane protein complexes following exposure to elevated temperatures (Gounaris *et al.,* 1984). A similar stabilisation of photosystem-I complex following membrane hydrogenation has also been described (Vigh, Gombos, Horvath, 1989).

The present study demonstrates that impairment of mitochondrial membrane protein function is directly related to the heat dose that causes heat death. Furthermore, thermotolerance can protect the proteins from heat damage, most likely as a result of the binding of heat shock proteins. In the present experimental work mitochondrial membrane lipid composition and fluidity were not measured. All flies were reared under the same conditions and so these parameters were not expected to differ between the various experimental and control groups, and in consequence would not have contributed to the different thermal sensitivities

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Appendix 1 .

Determination of LD_{50} and 95% confidence limits :

Notation:

$y_i \ldots y_n$ denotes available probits,

$x_i \ldots x_n$ denotes corresponding temperatures.

Calculate

$$\bar{x} = \left(\sum x\right)/n \quad \text{and} \quad \bar{y} = \left(\sum y\right)/n$$

$$Sxx = \left(\sum x^2\right) - \frac{\left(\sum x\right)^2}{n} \qquad Syy = \left(\sum y^2\right) - \frac{\left(\sum y\right)^2}{n} \qquad Sxy = \left(\sum xy\right) - \frac{\left(\sum x\right)\left(\sum y\right)}{n}.$$

Procedure

$y = a + b(x - \bar{x})$,

estimated intercept $= a = \bar{y}$,

estimated slope $= b = \frac{Sxy}{Sxx}$.

Median effective temperature (LD_{50}) $= m = \bar{x} + \frac{5 - a}{b}$,

95% confidence limits set by $m \pm$ s.e. (mean) $\times t$, where t is the tabulated student t value (95%) with n^{-2} degree of freedom .

$$\text{s.e. of } m = \sqrt{\frac{s^2}{b^2}\left[\frac{1}{n} + \left(\frac{M - x}{Sxx}\right)\right]^2},$$

$$\text{where } s^2 = \frac{1}{n-2}\left[Syy - \left(\frac{Sxy}{Sxx}\right)^2\right].$$