The Journal of Experimental Biology **198**, 2413–2421 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

THE DEVELOPMENT OF THERMOTOLERANCE PROTECTS BLOWFLY FLIGHT MUSCLE MITOCHONDRIAL FUNCTION FROM HEAT DAMAGE

RUKAYA EL-WADAWI AND KEN BOWLER*
Department of Biological Sciences, University of Durham, Durham City, DH1 3LE, UK

Accepted 21 July 1995

Summary

The LD_{50} of 10-day-old adult blowflies was determined to be 38.12±0.07 °C.

A transitory increase in heat resistance occurred following the exposure of adult blowflies to a sublethal heat shock at 36 °C. This thermotolerance was apparent 1 h after the application of the shock, was maximal 2–3 h later and had disappeared 6 h after exposure.

Oxidative phosphorylation by flight muscle mitochondria from control flies was impaired by an LD_{50} dose *in vivo* using both pyruvate+proline (P+P) and glycerol 3-phosphate (G3P) as substrates. Acceptor control (state III respiration/state IV respiration) was lost with G3P as substrate and so ADP:O ratios were not measurable.

The effect of experimental temperature *in vitro* on respiratory performance of mitochondria isolated from control and thermotolerant flies was also determined between 19 and 39 °C. State III respiration was markedly temperature-dependent in mitochondria from 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 was most marked for G3P. The effect of experimental temperature on state IV respiration was similar in mitochondria from control and thermotolerant flies with each substrate, but differed between the two substrates. With G3P as substrate, respiration rate rose with temperature with a Q_{10} of approximately 1.5; however, with P+P as substrate, the trend was for respiration rate to fall as experimental temperature rose.

Using G3P as substrate, acceptor control was demonstrable at 34 °C in some preparations of mitochondria from thermotolerant flies but not in those from control flies at that temperature. With P+P as substrates, acceptor control was demonstrable in mitochondria from both control and thermotolerant flies at all experimental temperatures.

Key words: heat injury, thermotolerance, mitochondria, oxidative phosphorylation, blowfly, *Calliphora vicina*.

Introduction

Heat injury is suffered by all organisms, as well as by cells in culture, at temperatures that are only slightly elevated above those normally experienced. The cause(s) of organismal and cellular heat death are not well understood. This is, in part, because heat has an all-pervasive influence on the cells of an organism (Bowler, 1987), but also because of the often-demonstrated hierarchical relationship that the organism is the most sensitive to heat, organ and tissue function are less sensitive, cells are less sensitive still, whilst individual proteins are least sensitive (Cossins and Bowler, 1976; Roti Roti and Laszlo, 1988). Jung (1991) has recently suggested, from data analysis using mathematical models, that cellular heat damage is a two-step process, in which primary damage is converted to permanent damage in a time-dependent fashion. This is consistent with the model for cellular heat injury proposed by Bowler (1987) suggesting that primary heat damage causes a variety of secondary and tertiary effects which, in a time-dependent manner, will cause irreversible injury and lead to death. A further complicating possibility in the search for the primary lesion is that its perturbation by heat may last only for the duration of the heat application, and so the resulting secondary and tertiary damage will be the cause of death. This allows for the possibility that secondary and tertiary damage may be cell- or organism-specific. The cause(s) of cellular and organism heat injury must be modifiable by acclimation to temperature, because acclimation alters the heat death points of organisms (Cossins and Bowler, 1987) and of cells in culture (Anderson *et al.* 1981; Culver and Gerner, 1982; Schmidt *et al.* 1984); furthermore, the primary lesion must be sensitive to the same heat dose as is responsible for heat death. As heat injury is experienced by all organisms (and cells), it is reasonable to consider that the primary lesion may be common

*Author for correspondence.

to them all, and an increasing body of evidence suggests that perturbation of membrane function may be its cause (Bowler *et al.* 1973; Bowler, 1987; Konings, 1988; Bowler and Manning, 1994; Yatvin and Cramp, 1993).

Many studies have shown that subjecting cells or organisms to a high, but sublethal, heat treatment causes a transitory increase in tolerance to a subsequent heat stress (e.g. Subject and Shyy, 1986; Rutledge *et al.* 1987). Such pre-shock treatments have been shown to evoke the enhanced synthesis of a specific suite of proteins (heat shock proteins, hsps), a response that is highly conserved and universal, from bacteria to mammalian cells (Schlesinger, 1990). It is suggested that the proteins of the various hsp families act to protect cellular proteins and nucleoproteins either from being heat-damaged, by binding to them, or by identifying damaged proteins and effecting a restoration of their proper folding (Pelham, 1986; Schlesinger, 1990; Laszlo, 1992), although the causative relationship between hsp production and thermotolerance remains in question (Smith and Yaffe, 1991).

The present study continues earlier work of Davison and Bowler (1971) and Bowler and Kashmeery (1981), who reported that *in vivo* heating of adult blowflies impaired the functional efficiency of flight muscle mitochondria. It is significant, in the earlier work, that in flies that recovered from the LD_{50} dose, both 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 is to demonstrate that heat shock induces thermotolerance in blowflies, and also that, in the thermotolerant state, mitochondrial function is protected from subsequent *in vivo* lethal heat exposure.

Materials and methods

Adult blowflies (*Calliphora vicina)* were bred in culture at 24 ˚C according to the methods described by Davison (1969).

Determination of heat death points

In adult blowflies, the heat death point has been shown to be age-dependent (Davison, 1969) so 10-day-old flies were used throughout the experiments. The LD_{50} was determined following the method described by Davison (1969). Batches of at least 100 flies were heated at eight temperatures between 36.5 and 40 ˚C for 40 min. The flies were then returned to their culture temperature (24±1 ˚C) for 2 days, after which the numbers of live flies were counted. These data were subjected to probit analysis and the LD_{50} dose determined (see Fig. 1).

Thermotolerance

Six hundred 10-day-old flies were subjected, in batches, to a sublethal thermal shock (36 ˚C for 40 min) and then returned to their culture temperature (24 ˚C). Samples of these flies were collected 1, 2, 3, 4, 5 and 6 h after thermal shock and exposed to the previously determined LD_{50} heat dose (38.1±0.1 ˚C for 40 min). An equal number of control flies (not pre-treated) were simultaneously subjected to the same LD_{50} heat dose. The batches of control and pre-treated flies were then returned to their culture temperature (24 ˚C) and the percentage remaining alive determined after 2 days (see Fig. 2).

Isolation of flight muscle mitochondria

Mitochondria from control and experimental flies were obtained using the following procedure. The thoraces from about 40 flies were removed and placed immediately into an ice-cold glass dish containing 2 ml of isolation medium [0.32 mol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 2 % (w/v) bovine serum albumin (BSA), 10 mmol l^{-1} Tris–HCl, pH 7.3 at 5 ˚C]. The thoraces were then gently pounded with a flat-bottomed glass rod, the resulting pulp was filtered through layers of washed muslin, and the filtrate was centrifuged at 100 ***g*** for 4 min at 4 ˚C. The supernatant was recentrifuged at 2200 ***g*** for 10 min at 4 ˚C, and the pellet was gently resuspended in 2 ml of isolation medium. This was recentrifuged at 2200 ***g*** for 10 min at 4 ˚C, and the resulting mitochondrial pellet was resuspended in 0.75 ml of ice-cold resuspension medium (0.15 mol l^{-1} KCl, 1 mmol l^{-1} EDTA in 10 mmol l^{-1} Tris–HCl, pH 7.3 at 5 ˚C). This procedure yielded a suspension containing between approximately 0.25 and 0.4 mg of mitochondrial protein in a 50 μl sample; previous work in our laboratory has shown that polarographic determination of oxidative phosphorylation is not dependent on mitochondrial protein concentration within this range. Mitochondria were isolated from control flies prior to, and from control and thermotolerant flies immediately after, an LD_{50} treatment. In a separate experiment investigating the effect of experimental temperature *in vitro* (19–39 ˚C) on oxidative phosphorylation, mitochondria were isolated from control flies and also from thermotolerant flies after 3 h at 24 ˚C following the exposure to 36 ˚C, to allow the full expression of thermotolerance, but without being subjected to an LD_{50} treatment (see Fig. 2).

Measurement of oxidative phosphorylation

Measurement of oxidative phosphorylation was carried out polarographically using a Rank oxygen electrode at 24 ˚C to determine oxygen consumption of isolated flight muscle mitochondria, using a reaction volume of 3 ml. When *rac* glycerol 3-phosphate (G3P) was used as the substrate, the reaction medium contained 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 30 mmol l^{-1} phosphate buffer, pH 7.3, and 20 mmol l^{-1} Tris–HCl, pH 7.3 at 24 ˚C. The final concentration of glycerol 3-phosphate was 33 mmol l^{-1}. When pyruvate+proline (P+P) were used as substrates, the reaction medium contained 154 mmol l^{-1} KCl, 30 mmol l^{-1} phosphate buffer, pH 7.3, and 0.4 % (w/v) BSA at 24 ˚C. The final concentrations of both pyruvate and proline were 2.5 mmol l^{-1}. In the study of the effect of experimental temperature on oxidative phosphorylation, the pH of reaction media was measured at the pertinent experimental temperature.

State III respiration was initiated by the addition of 0.5 μmol

samples of ADP in 30 mmol l^{-1} phosphate buffer (pH 6.8). Acceptor control (RCI) was measured using the method of Chance and Williams (1956). However, in some experimental situations, no respiratory 'cut-off' occurred following ADP addition; consequently, it was not possible to determine RCI *sensu* Chance and Williams (1956) and, in these cases, state IV respiration is assumed to be same as state III respiration, yielding an RCI of 1.

Protein determinations

The concentration of mitochondrial protein was determined using the Coomassie Brilliant Blue G250 method described by Bradford (1976) with BSA (fraction V) used as a standard.

Reagents

All reagents used were of the highest purity commercially available. Adenosine 5′-diphosphate (disodium salt), sodium pyruvate, proline, *rac* glycerol 3-phosphate, EDTA and BSA (fraction V) were obtained from Sigma Chemical Company. ADP was stored frozen at −20 °C in Tris–HCl buffer, pH 7.3.

Statistics

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.

Results

Fig. 1 shows the heat dose survival curve for 10-day-old adult blowflies from which the LD_{50} was determined to be 38.12±0.07 °C. This value is significantly lower than that determined for a similar stock of blowflies using the same protocol (40.9 °C; Davison, 1969). Fig. 2 shows the survival of control and heat-shocked flies after exposure to an LD_{50} heat dose. As can be seen, a time-dependent increase in survival occurred in the pre-treated group compared with the control group. Thermotolerance had developed 1 h after the heat shock, but maximal development required a period of 3–4 h at 24 °C, when a 30 % increase in survival above that of the equivalent control group was observed. There was then a progressive decay of the developed tolerance, which disappeared by 6 h after the termination of the heat shock.

Fig. 3 shows polarographic traces of the respiratory performance of mitochondria isolated from control (not LD_{50}-treated), LD_{50}-treated control and LD_{50}-treated thermotolerant flies, using both G3P (Fig. 3A) and P+P (Fig. 3B) as substrates. It should be emphasised that the traces obtained using the two substrates were from different samples of the same mitochondrial preparations. The addition of 0.5 μmol of ADP to control mitochondria initiated state III respiration, which changes to state IV respiration on the exhaustion of the added ADP. From these traces, respiratory rates, ADP:O ratios (the number of moles of ADP esterified to ATP for each gram atom of oxygen consumed) and acceptor control indices (state III rate/state IV rate) can be estimated; these data are shown in Table 1.

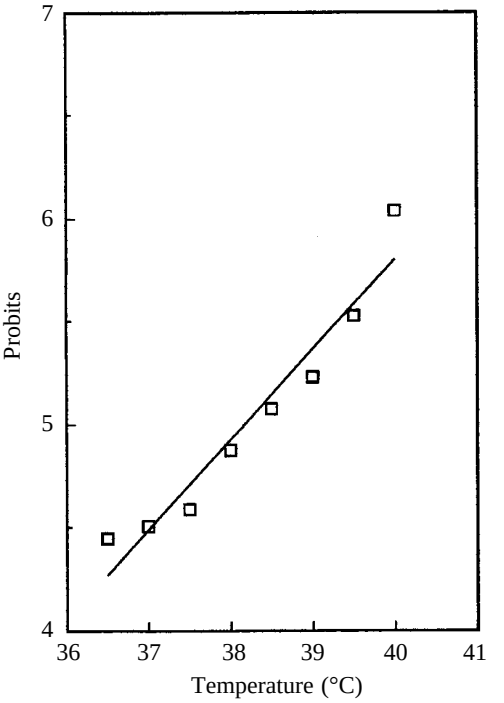

Fig. 1. Determination of the heat death point of 10-day-old adult blowflies. Batches of flies were exposed for 40 min at the eight temperatures shown. The number of survivors was determined after a 2 day recovery period at 24 °C. The percentage survival was converted into probits and the median lethal temperature (LD_{50}) determined (38.12±0.07 °C).

The rates of endogenous respiration (without added substrate) were usually not measurable, in agreement with earlier work on blowfly (Davison and Bowler, 1971; Bowler and Kashmeery, 1981) and housefly (van den Berg and Slater, 1962) flight muscle mitochondria. Respiration with G3P as the substrate in mitochondria from control flies was stimulated by the addition of ADP (RCI=2.18) with an ADP:O ratio of 2 approximately as expected. Using P+P as a substrate, control mitochondria show that respiration is also stimulated by the addition of ADP (RCI=5.75) and the ADP:O ratio is approximately 3 as expected. Mitochondria isolated from flies after an LD_{50} heat treatment have an impaired respiratory performance. With G3P as the substrate, state III respiration rate is significantly reduced, from 81.83±2.53 μg atoms O mg^{-1} protein h^{-1} in the control group to only 22.21±1.67 μg atoms O mg^{-1} protein h^{-1} (P=0.001), acceptor control is lost (RCI=1) and, consequently, ADP:O ratios were not measurable. With P+P as substrate, state III respiration rate is also significantly reduced (P=0.001), but state IV respiration rate is significantly increased compared with that of control mitochondria (P=0.05). This causes a sharp reduction in RCI from 5.75 to 1.93 (P=0.001) and a reduction in ADP:O is also observed (P=0.01).

Mitochondria isolated from thermotolerant flies subjected to the LD_{50} treatment had a respiratory performance similar to

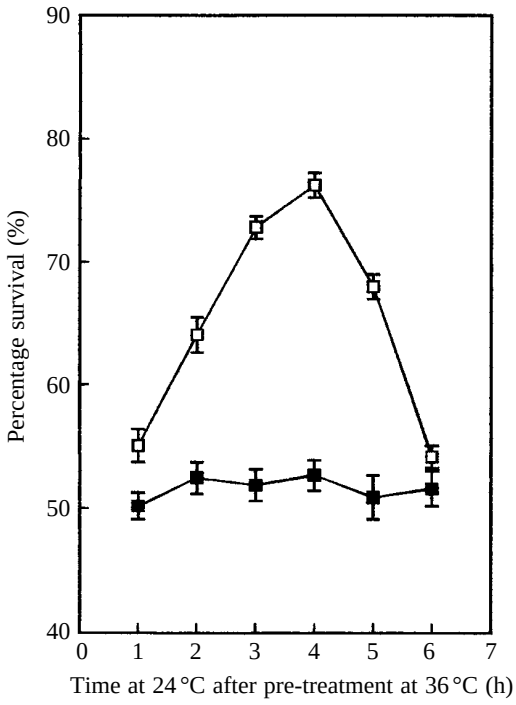

Fig. 2. The time course for the development of thermotolerance following pre-treatment for 40 min at 36 ˚C as determined from changes in the LD_{50} of 10-day-old blowflies. The pre-treated flies were returned to 24 ˚C and batches were taken at 1, 2, 3, 4, 5 and 6 h and their LD_{50} determined (open symbols) coincidentally with that of a batch of control flies that had not been pre-treated (filled symbols). Values are mean 1 ± S.E.M. (N=100).

that of the control mitochondria. The improvement over mitochondria from non-thermotolerant flies is pronounced when G3P is the substrate. Respiration rates for both state III, with added ADP, and substrate (pre-addition of ADP) are significantly lower than those of control mitochondria (P=0.001 in both cases), but state IV respiration rate is similar to that of control mitochondria. RCI values are not different, but ADP:O values are lower (P=0.01) than those of control mitochondria. Respiration with P+P as a substrate shows a different pattern of response. Substrate respiration rate is significantly increased in mitochondria following an LD_{50} treatment in both thermotolerant and non-thermotolerant control flies (P=0.001). Furthermore, state III respiration rate remains significantly lower in mitochondria from these groups compared with that of mitochondria from unheated flies (P=0.001). However, state IV respiration rate in the thermotolerant preparation is restored to the lower values obtained for control mitochondria. Values calculated for RCI correspondingly increased from 1.93±0.16 to 3.62±0.25 with thermotolerance; the latter value is still significantly lower than the value obtained for control mitochondria (P=0.001). In contrast to the use of G3P as a substrate, ADP:O was measurable in mitochondria from all treatment groups using P+P as substrate. Following an LD_{50} dose, the value obtained was significantly lower than that obtained for mitochondria from unheated flies (P=0.01), but in mitochondria from thermotolerant flies the ADP:O value does not differ from the control values.

Thus, the indices of the quality of the isolated mitochondria (RCI and ADP:O) are both significantly reduced by exposing the flies to an LD_{50} treatment, showing that oxidative phosphorylation is impaired. Table 1 shows that pre-treatment with a heat shock that induced thermotolerance protected the mitochondria from the damage caused by the subsequent LD_{50} dose; the RCI and ADP:O values for both substrate conditions are closer to the values from control mitochondria.

We then investigated whether induction of thermotolerance *in vivo* protected mitochondrial respiratory function against an *in vitro* exposure to high measuring temperature. Fig. 4 shows the effect of experimental temperature, from 19 to 39 ˚C, on state III respiration rate for mitochondria from control and thermotolerant flies. In control flies, with G3P as a substrate, respiration rate was significantly lower at 19 ˚C than at 24 ˚C (Q_{10}=2.95); no further significant increase in respiration rate occurred at 29 ˚C, but raising the measuring temperature to 34 ˚C caused a dramatic reduction in respiration rate. The results for mitochondria isolated from thermotolerant flies 3 h after a 36 ˚C heat shock showed a quite different pattern. State III respiration rate at 19 ˚C was significantly higher than that of control fly mitochondria (P=0.01), but was relatively independent of measuring temperature. With P+P as a substrate, state III respiration rates of control mitochondria were maximal at 24 ˚C and fell sharply at 29 ˚C, with a more gradual reduction between 29 ˚ and 39 ˚C. Here again, the state III respiration rates of thermotolerant mitochondria were less temperature-sensitive, showing no similar peak of respiration at 24 ˚C; indeed, respiratory rate at 29–39 ˚C was not significantly different from control values.

Fig. 5 shows the effect of experimental temperature on state IV respiration. Using G3P as a substrate, the effect of temperature was similar for both control and thermotolerant mitochondria. In controls, respiration rate rose with temperature with a Q_{10} of approximately 1.5 over the whole range, but in thermotolerant mitochondria, state IV respiration rate was relatively temperature-independent between 19 and 29 ˚C before following a similar relationship with temperature to the control data. With P+P as a substrate, the effect of temperature on respiration rates was also similar between the two groups, but respiration rates differed from those using G3P in that they tended to fall gradually and progressively with temperature increase.

RCI and ADP:O vaues for these experiments are summarized in Table 2. Considering first control mitochondria, a reduction in experimental temperature from 24 to 19 ˚C caused a significant fall in RCI with P+P as a substrate (P=0.01) without an effect on ADP:O, whereas with G3P no reduction occurred in RCI but ADP:O was lower (P=0.01). With G3P as a substrate, increasing temperature above 29 ˚C resulted in reduced RCI values compared with those at 24 ˚C, so that at 34 ˚C the value was significantly lower (P=0.001) and at 39 ˚C the value was not significantly different from 1. ADP:O values were significantly lower at 29 than at 24 ˚C

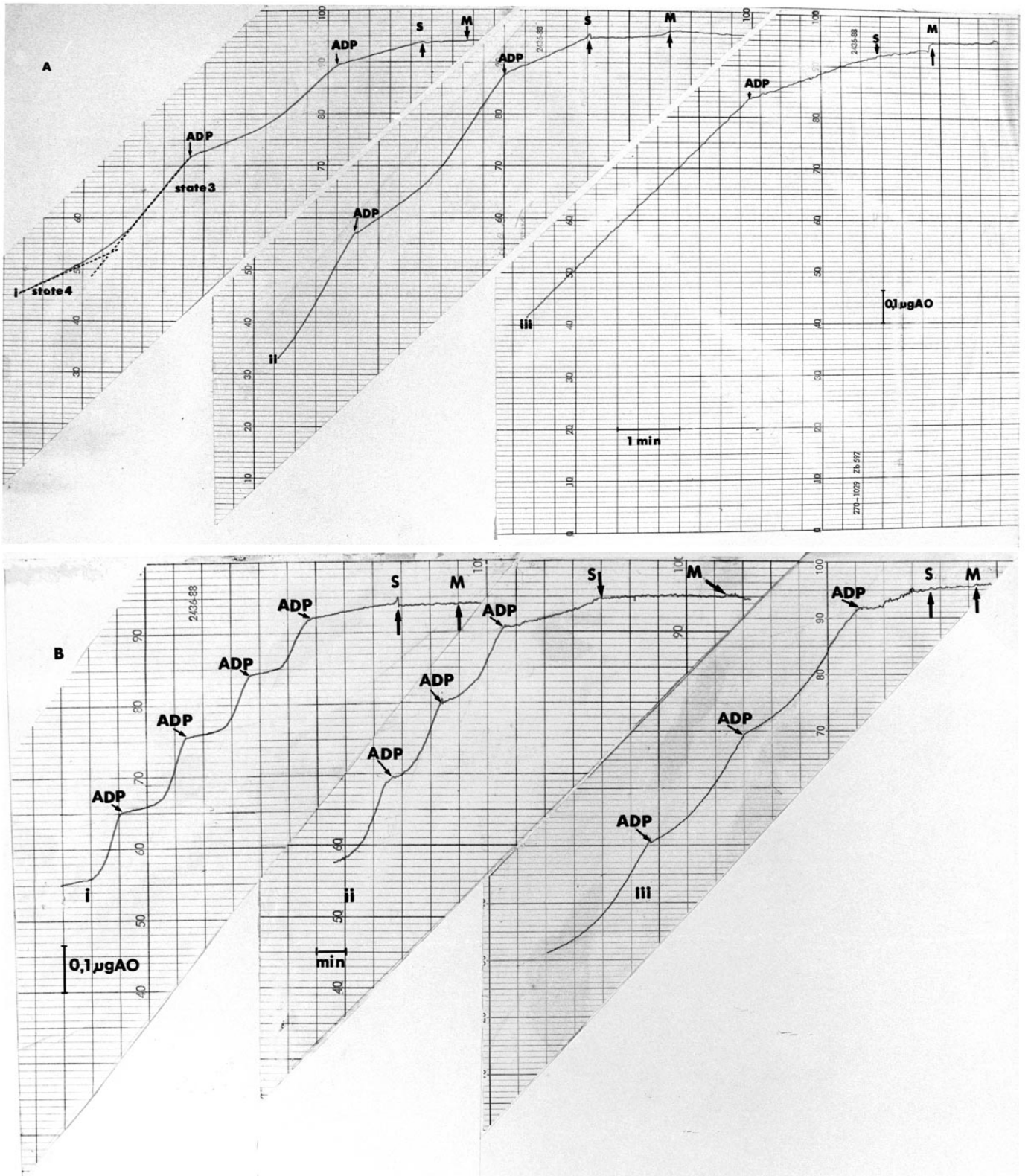

Fig. 3. Typical polarographic records obtained showing the rates of oxygen consumption of isolated flight muscle mitochondria at 24 ˚C. Arrows indicate additions of mitochondria (M), substrate (S) and 0.5 μmol of ADP (ADP). (A) Glycerol 3-phosphate as substrate; (B) pyruvate+proline as substrate. Trace i shows results from mitochondria from control flies, trace ii shows results from mitochondria isolated from the thermotolerant flies (3 h after exposure to 36 ˚C) after an *in vivo* LD_{50} heat dose, trace iii shows results from mitochondria isolated from flies that had not been pre-treated after an *in vivo* LD_{50} heat dose. The scale bars apply to all three traces for each substrate. The units of oxygen are expressed in microgram atoms. The dashed construction lines show state III and state IV respiration and indicate the method of determining the extra amount of oxygen consumed by the esterification of added ADP.

Table 1. *The effect of* in vivo *LD_{50} treatment on the respiratory efficiency at 24 °C of mitochondria isolated from normal and thermotolerant (3 h after a 40 min exposure to 36 °C) blowfly flight muscle*

		Respiratory rate						
Substrate	Treatment	Endogenous (without substrate)	With substrate	State III	State IV	RCI	ADP:O	Protein concentration (mg 50 μl^{-1})
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	NM	22.1±1.67***	22.21±1.67***	22.21±1.67***	1	NM	0.28±0.02
	LD_{50} thermotolerant	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 and proline	None	NM	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	NM	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} thermotolerant	NM	7.38±0.2***	23.99±1.27***	6.72±0.94	3.62±0.25***	2.70±0.08	0.31±0.02

Respiratory rate is measured in μg atoms O mg^{-1} protein h^{-1}.

The control mitochondria (none) were isolated from flies not subjected to LD_{50} treatment. Endogenous respiration is that measured without added substrate. State III is the respiratory rate after the addition of 0.5 μmol of ADP. State IV is the respiratory rate after the depletion of added ADP.

RCI, respiratory control index (acceptor control); ADP:O, the ratio of the number of moles of ADP esterified to ATP for each gram atom of oxygen consumed.

The mean value for LD_{50} control and LD_{50} thermotolerant groups were compared with those from control (none) mitochondria. Statistical differences were derived using Student's *t*-test or Whitney–Mann *U*-test as appropriate; *P<0.05; **P<0.01, ***P<0.001.

Values are means ± S.E.M.; N =20.

NM, not measured.

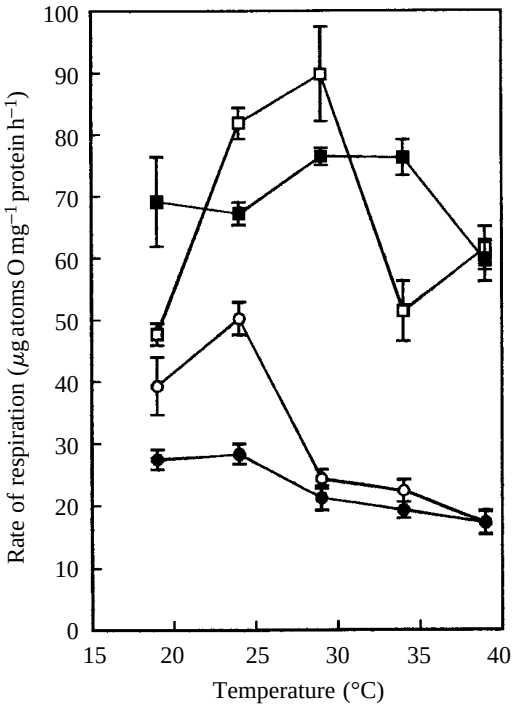

Fig. 4. The effect of experimental temperature on state III respiration rate of mitochondria isolated from flight muscle of control (open symbols) and thermotolerant (filled symbols) blowflies. The data are shown as means ± S.E.M. for respiration using glycerol 3-phosphate as a substrate (squares) and for pyruvate+proline (circles). See Table 2 for N values.

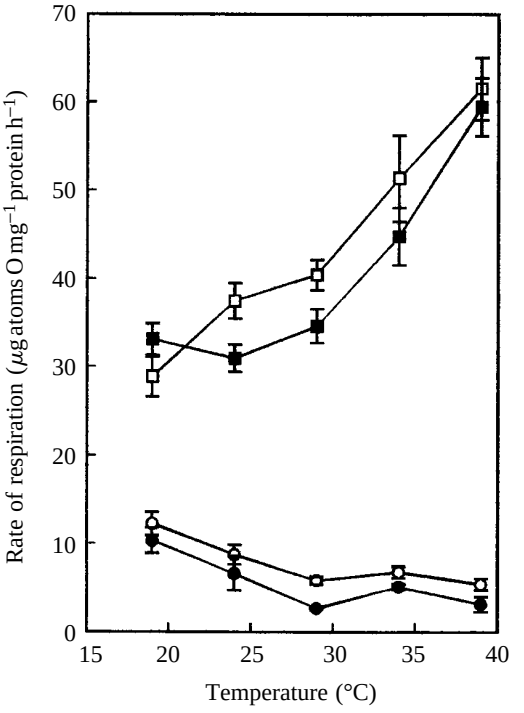

Fig. 5. The effect of temperature on state IV respiration rate of mitochondria isolated from flight muscle of control (open symbols) and thermotolerant (filled symbols) blowflies. The data are shown as means ± S.E.M. for respiration using glycerol 3-phosphate as a substrate (squares) and pyruvate+proline (circles). See Table 2 for N values.

Table 2. *The effect of experimental temperature on oxidative phosphorylation of mitochondria isolated from flight muscle of control (no heat treatment) and thermotolerant (TT) flies (flies given a 40 min pre-treatment at 36 °C followed by a 3 h period at 24 °C to allow the development of thermotolerance)*

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***	NM	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	NM	15
		TT	1	NM	13
	P + P	Control	3.15±0.4***	2.28±0.3**	15
		TT	2.28±0.29***	2.13±0.27**	13

The data were collected from about 40 different preparations of control and thermotolerant flies, with each preparation being used at two or three of the experimental temperatures.

Preparations were given glycerol 3-phosphate (G3P) or pyruvate + proline (P+P) as a substrate.

Values are means ± S.E.M.

RC1, ADP:O and NM are as in Table 1.

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.

(*P*=0.01) and were not measurable at higher experimental temperatures.

With P+P as a substrate, RCI values fell progressively from 5.75 at 24 ˚C to 4.1 at 29 ˚C (*P*=0.05), then to 3.57 at 34 ˚C and to 3.15 at 39 ˚C (*P*=0.001, in both cases). Compared with the value at 24 ˚C, ADP:O fell significantly with increasing experimental temperature (*P*=0.01 in all cases) and was still 2.28 at 39 ˚C (*P*=0.01).

The same analysis of data from mitochondria from flies made thermotolerant show that reducing measuring temperature to 19 ˚C does not affect the values obtained for either RCI or ADP:O when G3P is the substrate, but with P+P as a substrate, RCI is significantly lower (*P*=0.01) but ADP:O values are unaltered. In contrast to control mitochondria, RCI with G3P 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 2.23 and 2.24 obtained at 24 and 29 ˚C (*P*=0.01). However, at 39 ˚C, respiratory control was not demonstrable (RCI=1). The RCI of the thermotolerant mitochondria was significantly higher than that of control mitochondria at 34 ˚C with G3P (*P*=0.05). With G3P, an increase in experimental temperature caused a reduction in 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. With P+P as a substrate, the highest value for RCI (7.84) was obtained for thermotolerant mitochondria at 29 ˚C, which was significantly higher than the values obtained at 24 (*P*=0.01), and 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 value differ between the control and thermotolerant mitochondria when P+P were substrates.

Discussion

The LD_{50} we obtained for *C. vicina* is significantly lower than that obtained by Davison (1969) and Bowler and Kashmeery (1981). The cause for this difference is not clear, but may result from inadvertent selection of less resistant stock over the intervening period. The functional performance of the mitochondria from our 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 the LD_{50} value, the data obtained for the flies given an LD_{50} treatment were also effectively the same (Bowler and Kashmeery, 1981). Respiratory control was lost with G3P as substrate and reduced with P+P as a substrate. The reduction in state III respiration rate with both substrates, however, indicated either that the respiratory chain is inhibited by heating *in vivo* and/or that phosphorylation is inhibited whilst oxidative phosphorylation remains coupled. Floridini *et al.* (1987) have shown in mitochondria isolated from hyperthermia-treated Ehrlich ascites tumour cells that state III and state IV respiration rates are decreased, but RCI and phosphorylation remain normal compared with untreated cells. Their work suggests that a general inhibition of the electron transport chain occurred, 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) report that heating L5178YS cells resulted in a rapid reduction in ATP levels, a result confirmed by Jaing *et al.* (1991) from ^{31}P nuclear magnetic resonance studies during *in vivo* heating of a carcinoma.

The induction of thermal tolerance affords significant protection to the mitochondria during subsequent LD_{50} heating. In the case of G3P respiration, acceptor control is restored to control levels, although the ADP:O ratio remains significantly lower. State III respiration rate is restored to about 90 % of that of controls. In the case of P+P respiration, the protection afforded is to reduce state IV respiration rate, rather than increase state III respiration rate, which remains inhibited. RCI therefore remains significantly lower than control levels, but ADP:O values are restored to control levels. Lunec and Cresswell (1983) have shown that the development of thermotolerance protected the ability to maintain ATP levels in L5178YS cells subjected to a second heat treatment, a response that was dependent upon the time course of the appearance and decay of thermotolerance. Glycerol-3-phosphate dehydrogenase is thought to be the rate-limiting step for glycerol phosphate oxidation by the respiratory chain; this enzyme is allosterically stimulated by 10^{-5} mol l^{-1} Ca^{2+} (Hansford and Chappell, 1967). In earlier work, it was shown that whilst the catalytic properties of the enzyme were affected (e.g. 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 thermally stable (Bowler and Kashmeery, 1981). Several earlier studies indicate 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 G3P respiration in blowfly flight muscle mitochondria, which have a high capacity for respiration using this substrate. O'Brien *et al.* (1991) report that a number of mitochondrial oxidative enzymes from different species are inactivated by heat in a way that parallels the differences in organism body temperatures. Treatment of the preparations with chaotropic agents, which disrupt hydrophobic interactions between membrane lipids and proteins, markedly increased the thermal sensitivity of the proteins. These authors conclude that thermal inactivation of mitochondrial function results from the perturbation of the hydrophobic interactions between membrane lipids and proteins, a conclusion that 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).

The data presented in Figs 4 and 5 and Table 2 on the effect of *in vitro* temperature on state III respiration rates show that, in control mitochondria, both G3P and P+P respiration are temperature-sensitive. The induction of thermotolerance reduces the marked sensitivity to temperature shown by state III respiration rates with G3P as a substrate. It prevents the suppression of respiration at 19 °C and also prevents the sharp reduction seen at 34 °C when compared with rates at 24–29 °C for state III respiration, so that the rate at 34 °C is significantly higher in the thermotolerant group (P=0.001). With P+P as substrates, 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 rate (Fig. 5); however, the responses with G3P and P+P as substrates differed. G3P state IV respiration rate increased with rising experimental temperature in a predictable manner (Q_{10}=1.5) whereas with P+P as substrate, it fell. The reason for this differential effect of experimental temperature on state IV respiration with the two substrates is not evident.

Using G3P 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 P+P as a 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.

These data suggest not only that the processes that confer thermotolerance are present in mitochondria *in vivo* but also that they are conserved during isolation and are expressed during *in vitro* heating. This suggests that, if hsps are the protective agents, then they are probably transported into the mitochondria as a result of the pre-treatment shock. However, it cannot be discounted that hsps are synthesised within the mitochondria in response to heat shock. The hsp 60 family is the most likely family of stress proteins to be involved, because these proteins 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 *et al.* 1989). Indeed, members of this family of hsps are reported to be bound to the F_1F_o-ATPase of *Heliothus* mitochondria (Miller, 1987) and cytochrome *c* (Schlesinger, 1990). If thermotolerance causes increased levels of hsp 60, which binds to a variety of matrix proteins and the F_1F_o-ATPase, then these may be protected from the inactivating perturbation of heating. It is also possible that it is this association between mitochondrial proteins and hsps that modifies the temperature characteristics of the enzymes, as is evident in Fig. 4. Work is in progress to determine whether there is an increased level of hsp 60 present in thermotolerant blowfly mitochondria.

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.

References

ANDERSON, R. L., MINTON, K. W., LI, G. C. AND HAHN, G. M. (1981). Temperature-induced homeoviscous adaptation in Chinese hamster ovary cells. *Biochim. biophys. Acta* **641**, 334–348.

BOWLER, K. (1987). Cellular heat injury: are membranes involved? In *Temperature and Animal Cells* (ed. K. Bowler and B. J. Fuller). *Symp. Soc. exp. Biol.* **41**, 157–185.

BOWLER, K., DUNCAN, C. J., GLADWELL, R. T. AND DAVISON, T. F. (1973). Cellular heat injury. *Comp. Biochem. Physiol.* **45**, 441–450.

BOWLER, K. AND KASHMEERY, A. M. S. (1979). Recovery from heat injury in the blowfly *Calliphora erythrocephala. J. therm. Biol.* **4**, 197–202.

BOWLER, K. AND KASHMEERY, A. M. S. (1981). Effects of *in vivo* heating of blowflies on the oxidative capacity of flight muscle sarcosomes: a differential effect on glycerol 3-phosphate and pyruvate plus proline respiration. *J. therm. Biol.* **6**, 11–18.

BOWLER, K. AND MANNING, R. (1994). Membranes as the critical targets in cellular heat injury and resistance adaptation. In *Temperature Adaptation of Biological Membranes* (ed. A. R. Cossins), pp. 185–204. London: Portland Press.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye-binding. *Analyt. Biochem.* **72**, 248–254.

CHANCE, B. AND WILLIAMS, G. C. (1956). The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.* **17**, 65–134.

CHRISTIANSEN, E. N. AND KVAMME, E. (1969). Effects of thermal treatment on mitochondria of brain, liver and ascites cells. *Acta physiol. scand.* **76**, 472–484.

COSSINS, A. R. AND BOWLER, K. (1976). Resistance adaptation of the freshwater crayfish and thermal inactivation of membrane-bound enzymes. *J. comp. Physiol.* **111**, 15–24.

COSSINS, A. R. AND BOWLER, K. (1987). *Temperature Biology of Animals*. London: Chapman & Hall.

CULVER, P. S. AND GERNER, E. W. (1982). Temperature acclimation and specific cellular components in the regulation of the thermal sensitivity of mammalian cells. In *Cancer Therapy by Hyperthermia, Drugs and Radiation* (ed. L. Dethelfesen), pp. 99–101. Bethesda, MD: US Department of Health and Sciences.

DAVISON, T. F. (1969). Changes in temperature tolerance during the life cycle of *Calliphora erythrocephala. J. Insect Physiol.* **154**, 977–988.

DAVISON, T. F. AND BOWLER, K. (1971). Changes in the functional efficiency of flight muscle sarcosomes during heat death of adult *Calliphora erythrocephala*. *J. cell. Physiol.* **78**, 37–48.

FLORIDINI, A., NISTA, A., PAGGI, M. G., PELLEGRINI, L., BAGNATO, A., FANCHIULLI, M. AND CAPUTO, A. (1987). Effect of hyperthermia on electron transport in Ehrlich ascites tumor mitochondria. *Exp. molec. Pathol.* **46**, 279–293.

HANSFORD, R. G. AND CHAPPELL, J. B. (1967). The effect of Ca^{2+} on the oxidation of glycerol phosphate by blowfly flight muscle mitochondria. *Biochem. biophys. Res. Commun.* **27**, 686–692.

JAING, Q., CHOPP, M., KOVICH, K., JOHNSON, C. AND HETZEL, F. W. (1991). Dose-dependent thermal response of tumor pH and energy metabolism evaluated by *in vivo* ^{31}P NMR spectroscopy and microelectrodes. *Radiat. Res.* **127**, 177–183.

JUNG, H. (1991). A generalized concept for cell killing by heat. Effect of chronically induced thermotolerance. *Radiat. Res.* **127**, 235–242.

KONINGS, A. W. T. (1988). Membranes as targets for hyperthermic cell killing. *Recent Results Cancer Res.* **109**, 9–21.

LASZLO, A. (1992). The thermoresistant state: protection from initial damage or better repair? *Expl Cell Res.* **202**, 519–531.

LUNEC, J. AND CRESSWELL, S. R. (1983). Heat-induced thermotolerance in energy metabolism of mammalian cells. *Radiat. Res.* **93**, 588–597.

MCMULLIN, T. W. AND HALLBERG, R. L. (1987). A normal mitochondrial protein is selectively synthesised and accumulated during heat shock in *Tetrahymena thermophillia. Molec. cell. Biol.* **7**, 4414–4423.

MILLER, S. G. (1987). Association of sperm specific protein with the mitochondrial F_oF_1ATPase in *Heliothis*. *Insect Biochem.* **17**, 417–432.

MORRIS, R. O. AND KING, T. E. (1962). Thermal denaturation of the heart muscle preparation with respect to its capacity for DPNH oxidation. *Biochemistry, N.Y.* **1**, 1017–1024.

O'BRIEN, J., DAHLHOFF, E. AND SOMERO, G. N. (1991). Thermal resistance of mitochondrial respiration: hydrophobic interactions of membrane proteins may limit thermal resistance. *Physiol. Zool.* **64**, 1509–1526.

OSTERMANN, J., HORWICH, A. L., NEUPERT, W. AND HARTL, F. U. (1989). Protein folding in mitochondria requires complex formation of hsp60 and ATP hydrolysis. *Nature* **341**, 125–130.

PELHAM, H. R. (1986). Speculations on the functions of the major heat shock protein and glucose-regulated proteins. *Cell* **46**, 959–961.

ROTI ROTI, J. L. AND LASZLO, A. (1988). The effect of hyperthermia on cellular molecules. In *Hyperthermia and Oncology* (ed. M. Urano and E. Douple), pp. 13–56. Utrecht: VSP.

RUTLEDGE, P. S., SPOTILA, J. R. AND EASTON, D. P. (1987). Heat hardening in response to two types of heat shock in the lungless salamanders *Eurycea bislineata* and *Desmognathus ochrophaelus. J. therm. Biol.* **12**, 235–241.

SCHLESINGER, M. J. (1990). Heat shock proteins. *J. biol. Chem.* **265**, 12111–12114.

SCHMIDT, J., LAUDIEN, H. AND BOWLER, K. (1984). Acute adjustments to high temperature in FHM cells from *Pimephales promelas* (Pices, Cyprinidae). *Comp. Biochem. Physiol.* **78**A, 823–828.

SMITH, B. J. AND YAFFE, M. P. (1991). Uncoupling thermotolerance from the induction of heat shock proteins. *Proc. natn. Acad. Sci. U.S.A.* **88**, 11091–11094.

SUBJECT, J. R. AND SHYY, T. T. (1986). Stress protein systems of mammalian cells. *Am. J. Physiol.* **250**, C1–C17.

VAN DEN BERG, S. G. AND SLATER, E. C. (1962). The respiratory activity and permeability of housefly sarcosomes. *Biochem. J.* **93**, 128–136.

YATVIN, M. B. AND CRAMP, W. A. (1993). Role of cellular membranes in hyperthermia: some observations and theories reviewed. *Int J. Hyperthermia* **9**, 165–186.