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THERMAL RESPONSE OF LIVER MITOCHONDRIAL MEMBRANES OF THE HETEROTHERMIC BAT (*MINIOPTERUS SCHREIBERSII*) IN SUMMER AND WINTER

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Abstract—1. The thermal responses of liver mitochondrial membranes of heterothermic bats (*M. schreibersii*) during summer and winter were compared with those of normothermic laboratory mice (*Mus musculus*).

2. Bat mitochondrial membranes showed a constant Arrhenius E_a for succinate oxidase and succinate:cytochrome *c* reductase and no change in the temperature coefficient of the order parameter of spin label over the temperature range 7–40°C. For the mouse these enzymes showed an increase in E_a below about 24°C and a change in molecular ordering at the same temperature.

membranes of bats collected in summer and winter.

4. The thermal response of the bat liver mitochondrial membranes is consistent with the heterothermic behaviour of this species.

Key Word Index—Arrhenius activation energy; activity/torpor; summer/winter; *Miniopterus schreibersii*; *Mus musculus*.

INTRODUCTION

Mammals are generally considered to be homeothermic, but an ever increasing number of species, particularly in the orders Insectivora, Rodentia, Chiroptera and Marsupialia have been shown to undergo torpor and lower their body temperatures (T_b) below 10°C. Among the Microchiroptera, members of the families Vespertilionidae and Rhinolophidae exhibit not only winter hibernation (Lyman, 1970) but also diurnal heterothermy during summer when ambient temperatures (T_a) are below their thermoneutral zone (Davis, 1970). These species are therefore described as heterothermic endotherms (Twente and Twente, 1964) because they retain the ability to spontaneously arouse, using endogenous heat production, even when T_b approaches 0°C (Henshaw, 1970; Lyman, 1970). In bats, the rate of rewarming during arousal is particularly rapid (Lyman, 1970). In this study we have examined some functional and structural aspects of liver mitochondrial membranes in relation to the heterothermic behaviour of an Australian Vespertilionid, the common bent-wing bat *Miniopterus schreibersii*.

Heat production ultimately depends on mitochondrial function, and recent studies have shown a correlation between the thermal response of heart and liver mitocondrial membrane lipids and seasonal variations in the T_b of mammalian hibernators. The membrane lipids of liver mitochondria from summer ground squirrels (Keith *et al.*, 1975) and those of blood cells from summer hedgehogs (Augee *et al.*, 1979) show a change in molecular order at about 20–23°C, similar to that found in obligate homeothermic mammals (Raison *et al.*, 1971). This is coincident with a change in the kinetics of membrane-associated enzymes such as succinate oxidase, which show an increase in the Arrhenius activation energy (E_a) below 23°C in rabbits and rats (McMurchie *et al.*, 1973; McMurchie and Raison, 1979) and active summer ground squirrels (Raison and Lyons, 1971). During hibernation in winter however, liver mitochondria of ground squirrels show no change in the E_a of succinate oxidase (Raison and Lyons, 1971) and no change in molecular ordering of membrane lipids (Keith *et al.*, 1975) over the range 5–35°C. The change in E_a of enzyme function is interpreted as a manifestation of a change in ordering of a small number of lipid molecules (Raison *et al.*, 1981) rather than a solidification of the bulk lipids of the membrane (Cossins, 1981). Not all membrane-associated enzymes show this response to hibernation (Charnock, 1978). Myocardial ATPase shows an increase in E_a below about 15°C from both active and torpid squirrels although changes occur in the thermal response of the plasma membrane lipids during torpor (Charnock *et al.*, 1980).

In endotherms the temperature at which the changes occur in the E_a of succinate oxidase activity and mitochondrial membrane lipid ordering, approx-

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Abbreviations: E_a, Arrhenius activation energy; T_b, body temperature; T_a, ambient temperature; ESR, electron spin resonance spectroscopy; S_n, order parameter of spin label; DPH, 1,6-diphenyl-1,3,5-hexatriene; HEPES, *N*-2 hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid; EDTA, ethylenediaminetetra-acetic acid.

imates the minimal T_b for survival during hypothermia (Raison *et al.*, 1981). However, many of the small mammalian species in the orders mentioned above, and particularly the known hibernators, routinely survive prolonged T_b well below 20°C without impaired function. These hibernators have marked seasonal changes in the thermal response of their mitochondrial or blood cell membranes which occur, in contrast with ectotherms (Wodtke, 1976; Van den Thillart and Modderkolk, 1978), independently of exposure to low temperature (Augee *et al.*, 1979). This suggests that lowering the temperature for the change in lipid ordering is a necessary feature of the preparation required by endotherms to survive low T_b. Since these changes are reversed at the end of winter, it would appear that there is some advantage to mammalian hibernators in not maintaining the thermal properties of the "winter-type" membrane during the summer when the animals are active.

This raises the question of what seasonal changes occur in the membranes of the endothermic *M. schreibersii*, which undergoes daily torpor even in summer. We have examined the thermal properties of liver mitochondrial membranes of this species during winter (June–July in Australia), the period of minimal activity, and during summer, the period of high activity (Dwyer, 1964), and found that for both seasons membrane structure and function resemble that of torpid hibernators.

MATERIALS AND METHODS

Animals

Bent-wing bats (*Miniopterus schreibersii blepotis*, Vespertilionidae) were captured in a cave in the Sydney area and were kept in the laboratory at temperatures close to that of the cave. They were killed within 2 days of capture. In the winter (June–July, $n = 11$) most of the bats were torpid on the wall of the cave at a T_a of about 14°C ($T_b = 16$–17°C) and only capable of clumsy crawling. A few specimens flew when disturbed by noise. In the summer (February–March, $n = 11$) the bats were also found in shallow torpor at a T_a of about 20°C with a larger proportion of the bats alert and many flew when disturbed by light. All specimens of the summer bats were torpid ($T_b = 22$–29°C) the day after capture when maintained in the laboratory at 20°C. The mean body weight was 13.5 g and the liver weight was between 0.43 and 0.49 g. Mice (*Mus musculus*) were obtained from a laboratory colony at Macquarie University. They were maintained at a T_a of 25°C with water and commercial pellet diet *ad libitum* and therefore normothermic (Hudson and Scott, 1979; Webb *et al.*, 1982).

Isolation of mitochondria

Bats and mice were decapitated and the livers were removed immediately and placed in ice-cold medium consisting of: 250 mM sucrose, 2 mM HEPES, 0.5 mM EDTA and 0.5 mg ml^{-1} bovine serum albumin (pH 7.4). The liver was homogenized with a glass–Teflon homogenizer using about 5 ml of medium per g of tissue. The homogenate was centrifuged at 750 *g* for 7 min and the mitochondria obtained from the supernatant by centrifugation at 12,100 *g* for 7 min. The mitochondria were washed once and sedimented by centrifugation at 8700 *g* for 15 min. For the bats two to three livers were pooled to obtain sufficient mitochondria for both the succinate:cytochrome *c* reductase and for the succinate oxidase assays. For mice sufficient mitochondria were obtained from individual livers.

Lipid extractions

Mitochondria were extracted with $CHCl_3$:CH_3OH (2:1, v/v) and protein was removed from the total lipid extract by adsorption to Sephadex G-25 as previously described (McMurchie and Raison, 1975).

Enzyme activity

Succinate oxidase activity was measured polarographically in 3 ml of medium containing: 100 mM sucrose, 50 mM KCl, 15 mM HEPES, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mg ml^{-1} of bovine serum albumin, 5 mM succinate adjusted to pH 7.4 with KOH at the temperature of activity measurement. The reaction usually contained about 3 mg of mitochondrial protein and sufficient ADP was added to determine several cycles of both State 3 and State 4 rates.

Succinate:cytochrome *c* reductase activity was measured after mitochondria were swollen in 10 mM phosphate buffer (pH 7.2) for 20 min at 22°C. The reduction of cytochrome *c* was measured at 550 nm in a reaction mixture containing: 50 mM phosphate (pH 7.2), 1 mM potassium cyanide, 80 μM cytochrome *c*, 10 mM succinate and mitochondria equivalent to about 1 μg of protein per ml. Protein was estimated by the method of Lowry *et al.* (1951).

Electron spin resonance spectroscopy (ESR)

Mitochondrial membranes were labelled with 3-oxazolidinyloxy-2-(4-carboxypropyl)-2-tridecyl-4,4-dimethyl (5NS) obtained from Synvar Assoc. (Palo Alto, Calif., U.S.A.). The spin label was mixed with aqueous suspensions of the membrane preparations in the proportion of approx. 1 mol of label per 100 mol of membrane lipid. Spectra were recorded with a Varian E4 spectrometer fitted with a temperature-controlled sample holder (Raison and McMurchie, 1974), and the order parameter (S_n) of spin label was obtained from measurements of the hyperfine splittings of the first derivative absorption spectra, as described by Sauerheber *et al.* (1977), and calculated as described by Gaffney (1976).

Fluorescence measurements

Total lipids from the mitochondria (1200 μg in $CHCl_3$) were mixed with 1 nmol of 1,6-diphenyl-1,3,5-hexatriene (DPH) and concentrated under nitrogen and freed from solvent under vacuum. The mixture was dispersed by sonication for about 2 min at 40°C in 0.2 M Tris–acetate buffer (pH 7.2) containing 2 mM EDTA. Fluorescence polarization was measured with an excitation wavelength of 365 nm (slit 1 mm) and an emission wavelength of 410 nm (slit 3 mm) and was calculated as described by Brasitus *et al.* (1980) from the fluorescence anisotropy r, and the anisotropy parameter $[(r_0/r) - 1]^{-1}$, where $r = (I - I_\perp)/(I_\parallel + 2I_\perp)$ and $r_0 = 0.362$. The anisotropy parameter varies directly with the relaxation time of the probe and therefore is related inversely to lipid fluidity.

Statistical analysis

For the determination of a change in slope the correlation coefficient (r^2) and the residual sum of squares was calculated for all possible combinations of points fitted to two straight lines from the lower to the upper temperature extremes. A change in slope was considered to occur at the minimum for the sum of the residual sum of squares of the two straight lines. Tests for the significance of paired observations were made using the Student t-test.

RESULTS

The liver mitochondria used in this study were freshly isolated and exhibited respiratory control. The effect of temperature on the State 3 rate of succinate oxidase activity in mitochondria from both

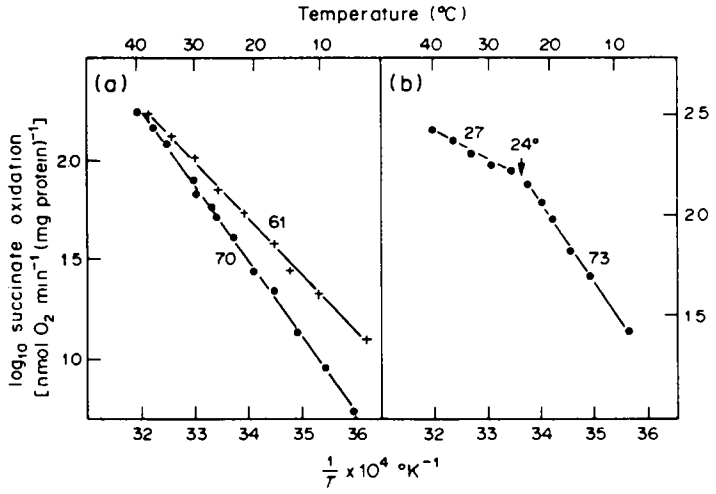

Fig. 1. Arrhenius plots of State 3, succinate oxidase activity of: (a) liver mitochondria from the bat *M. schreibersii* in summer (●) and winter (+); and (b) liver mitochondria from the mouse *Mus musculus*. The numbers adjacent to the lines are the Arrhenius activation energies (E_a) in kJ mol^{-1}.

summer and winter bats is shown in Fig. 1a as Arrhenius-type plots. This enzyme system shows a constant E_a over the temperature range 5–40°C, for both groups of bats, contrasting with the increase in E_a below 24°C which occurs in plots of mouse succinate oxidase activity (Fig. 1b).

There is considerable controversy as to whether data on the variation of the log of enzyme activity as a function of the reciprocal of absolute temperature should be considered as one, or two, linear-related functions (as shown in Fig. 1a, b) or as a continuously variable function (as in Fig. 2). In Fig. 2 we have fitted the data shown in Fig. 1b to the equation described by Silvius and McElhaney (1980). This shows that the E_a (slope of the tangent to the curve) increases continuously as the temperature decreases. The best fit to the data was obtained using a value of 102.4 kJ mol^{-1} for the enthalpy of the transition (ΔH_{21}) of the enzyme between the high and low activity states and a value of 293°K (20°C) for the temperature (T_0) at which the enzyme is equally distributed between the two states (Silvius and McElhaney, 1980). In defined lipid mixtures (Silvius and McElhaney, 1980) T_0 was unusually about 3–8°C below the exothermic transition temperature of the lipids. Thus, irrespective of how the data is presented or treated it is apparent that E_a of succinate oxidase (and succinate:cytochrome *c* reductase as shown in Fig. 3b) of liver mitochondria of mouse, increases below about 23°C and is consistent with the view that this is due to a change in lipid ordering.

Differences between bats and mice were also apparent when the temperature response of succinate:cytochrome *c* reductase was compared. As shown in Fig. 3, the E_a for this enzyme for both summer and winter bats was constant over the temperature range 5–40°C but the same enzyme system from the mouse showed an increase in E_a below 23°C.

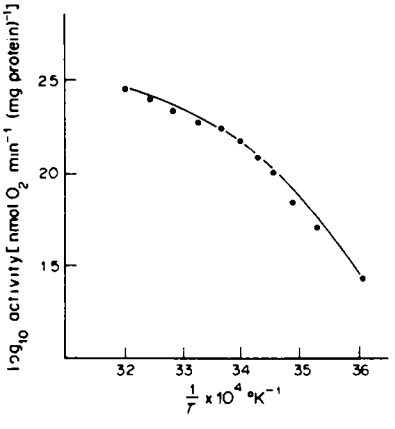

Fig. 2. Relationship between the log of succinate oxidase activity of mouse liver mitochondria as a function of the reciprocal of absolute temperature. The data were fitted to the equation: $\log \text{rate} = 1/2.303\,[K + F(T) - \ln\{1 + \exp\{(\Delta H_{21}/R)/(1/T - 1/T_0)\}\}]$ (Silvius and McElhaney, 1980). The constants were $K = 0.25$, $F = 0.017$, $\Delta H_{21} = 102.4$ kJ mol^{-1} and $T_0 = 293$ K (20°C), and the residual sum of squares for the fit was 0.0078.

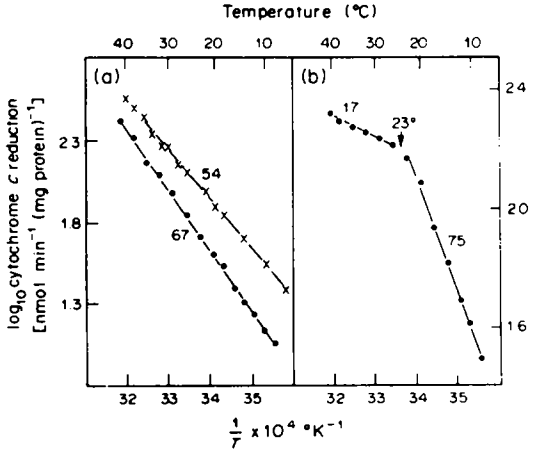

Fig. 3. Arrhenius plots of succinate:cytochrome *c* reductase activity for mitochondrial membranes of: (a) the bat *M. schreibersii* in summer (●) and winter (+); and (b) the mouse *Mus musculus*. The numbers adjacent to the lines are the Arrhenius activation energies (E_a) in kJ mol^{-1}.

Table 1. Comparison of the Arrhenius E_a and transition temperature for succinate oxidase and succinate:cytochrome *c* reductase from mouse and bat liver mitochondria

Enzyme	Animal	Season	Transition temperature (°C)	E_a (kJ mol^{-1}) E_{a_1}	E_{a_2}
Succinate oxidase	*Mus musculus*		24.1 ± 0.5	26.7 ± 1.9	62.7 ± 3.5 (6)
	M. schreibersii	Summer	—	68.3 ± 1.4 (3)	
	M. schreibersii	Winter	—	64.0 ± 3.2 (4)	
Succinate : cytochrome *c* reductase	*Mus musculus*		22.6 ± 0.7	28.4 ± 2.4	70.6 ± 5.8 (6)
	M. schreibersii	Summer	—	62.3 ± 5.0 (3)	
	M. schreibersii	Winter	—	47.6 ± 3.7 (4)	

E_{a_1} and E_{a_2} are the Arrhenius activation energies above and below the transition temperature, respectively. The values are the mean ± SEM for the number of mitochondrial preparations shown in parentheses.

As shown in Table 1 the temperature for the change in the E_a for the membrane-associated enzyme systems of the mouse is 24.1 ± 0.5°C (succinate oxidase) and 22.6 ± 0.7°C (succinate:cytochrome *c* reductase). These temperatures are not significantly different ($P < 0.25$). It should also be noted that the absolute value for the specific activity of both succinate:cytochrome *c* reductase and succinate oxidase for mice and bats are similar (Figs 1 and 3). The plots shown in Figs 1 and 3 are for individual mitochondrial preparations. The data for all preparations are summarized in Table 1. Although the E_a for both succinate oxidase ($P < 0.25$) and succinate:cytochrome *c* reductase ($P < 0.05$) for the winter bats is slightly lower than for the summer bats this difference is hardly significant.

An increase in the E_a of membrane-associated enzyme systems of mitochondria at low temperatures has previously been shown to be induced by an alteration in molecular ordering of membrane lipids (Raison *et al.*, 1971). For other enzyme systems such increases have been attributed to a temperature-dependent change in the affinity of the enzyme for substrate (K_m) or to an inhibition by substrate at low temperature (Silvius and McElhaney, 1980). To investigate this, the K_m with respect to succinate for mouse mitochondrial succinate:cytochrome *c* reductase was determined at temperatures above and below 23°C. As shown in the Lineweaver–Burk plots in Fig. 4, the K_m varied only slightly for reactions at 30 and 15°C (0.26 to 0.30 mM, respectively) and at 15°C there was no evidence of substrate inhibition at the concentration of succinate (10 mM) used in the assay. Thus the increase in E_a cannot be attributed to an alteration in enzyme affinity or substrate inhibition at low temperature, and is therefore attributed to a change in the confirmation of the active site of the enzyme. This could be due to either a direct effect of temperature on the enzyme protein, as with ATPase (Charnock *et al.*, 1980), or induced by a change in ordering of associated membrane lipids. As shown in Fig. 5, the order parameter (S_n) for the lipids in mouse mitochondrial membranes determined by ESR, showed an increase in temperature coefficient below 25°C, approximately the temperature at which the E_a of the liver enzyme systems changed. In contrast, with mitochondria from a summer bat, this parameter is a linear function with temperature.

The fluidity of membrane lipids usually decreases with decreasing temperature (Sinensky, 1974) and it was therefore of interest to compare the effect of decreasing temperature on the fluidity of the mitochondrial membranes of the bat with that of the mouse. Comparative measurements of lipid fluidity were made using the anisotropy parameter derived from fluorescence polarization of DPH where an increase in the value indicates a decrease in fluidity. As shown in Table 2 the anisotropy parameter of the membrane lipids of the mouse and summer and winter bats at 40°C was half that at 10°C. Although the anisotropy parameter of the membrane lipids of the winter bats was slightly greater than that of the summer bats at both temperatures this difference was not significant ($P < 0.25$). The anisotropy parameter for mouse membrane lipids however was significantly

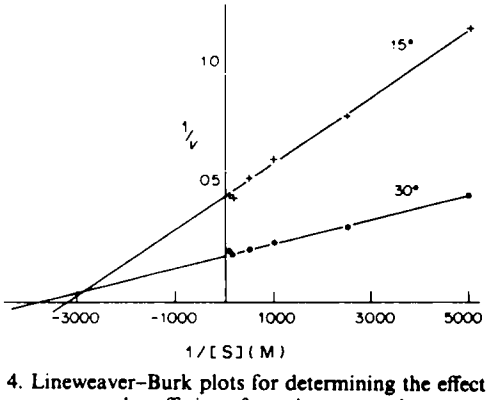

Fig. 4. Lineweaver–Burk plots for determining the effect of temperature on the affinity of succinate:cytochrome *c* reductase for substrate using mouse liver mitochondrial preparations.

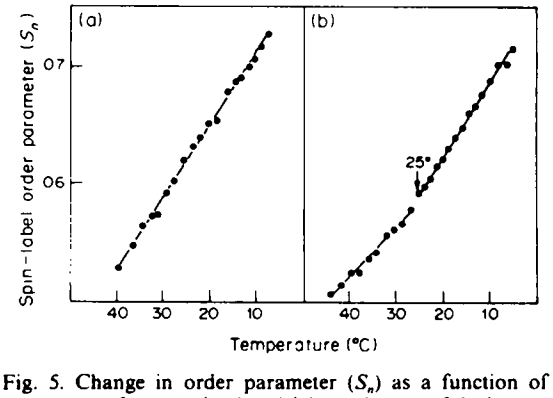

Fig. 5. Change in order parameter (S_n) as a function of temperature for: (a) mitochondrial membranes of the bat *M. schreibersii* in summer; and (b) mitochondrial membranes of the mouse *Mus musculus*. Each point represents the means of three determinations.

Table 2. Fluorescence polarization of liver mitochondrial total lipids

Animals	Temperature (°C) 10	40
M. schreibersii		
(Summer)	3.086 ± 0.337	1.434 ± 0.113 (4)
(Winter)	2.56 ± 0.193	1.359 ± 0.038 (4)
Mus musculus	1.554 ± 0.033	0.782 ± 0.014 (7)

The values are the means of the anisotropy parameter $[(r_0/r) - 1]^{-1}$ ± SEM for the number of determinations shown in parentheses.

lower than for that of the bat ($P < 0.001$) at both 40 and 10°C.

DISCUSSION

Differences in the thermal response of mitochondrial membranes of the heterothermic bat and normothermic mouse with regard to both structure and function are revealed by the temperature studies. For the mouse the increase in the E_a of succinate oxidase and succinate:cytochrome *c* reductase (Figs 1 and 3), and the change in molecular ordering of membrane lipids (Figs 2 and 5), occurs at about 23°C as previously found for the mitochondrial membranes of other homeotherms (Raison and McMurchie, 1974). This contrasts with the constant E_a for the same enzymes and the lack of a change in molecular ordering of lipids with the mitochondrial membranes of the bat over the same temperature range of 5–40°C. Since it was found that E_a above the transition temperature increases as the transition temperature is lowered (McMurchie and Raison, 1979) the constant, but greater, E_a for the membranes of the bat is interpreted to indicate that the transition in structure and function for this membrane is below 5°C. Thus in respect to the thermal response of the mitochondrial membranes, the bat resembles that of ground squirrels during torpor (Raison and Lyons, 1971; Keith *et al.*, 1975). However in contrast with the mitochondrial membranes of the seasonal mammalian hibernators, which alter their membranes during their active summer period to the pattern typical of obligate homeotherms, the membranes of the heterothermic bat exhibit the same thermal response in both seasons investigated. With regard to fluidity, as measured by fluorescence polarization (Table 2), the membrane lipids of the bat differ from those of ectotherms which in general are more fluid than those of normothermic mammals. For the ectotherms, membrane lipid fluidity increases when cold adapted (Cossins and Prosser, 1978). For the bat, the membrane lipids were relatively less fluid than those of the normothermic mouse and did not change significantly on a seasonal basis (Table 2). It should be stressed however, that membrane lipid fluidity inferred from measurements using added probe represents only a measure of the micro-environment in the immediate vicinity of the probe rather than properties of the bulk lipids (Hare *et al.*, 1979). Thus while comparisons of the thermal response of membrane lipids determined by the use of appropriate probes, provide useful information, comparisons of the absolute values of label motion, ordering or polarization are of doubtful physiological significance (Kleinfeld *et al.*, 1981).

Most mammalian hibernators are homeothermic during summer and resist torpor at least during their breeding season (Hoffman, 1964). Insectivorous bats are an exception to this, since their T_b falls during daily inactive periods independent of any season (Hock; 1951) and latitude (McNab, 1969). The species studied in this paper, *M. schreibersii*, undergoes daily torpor even during the Australian summer, in temperate as well as tropical regions (Kulzer *et al.*, 1970). The fact that there is no significant difference in the thermal response of the mitochondrial membranes of the bats collected during a period of high daytime activity in summer and those collected during the period of least activity in winter (Figs 1 and 3) is further evidence that they are heterothermic throughout the year. In this respect they differ from other mammalian, seasonal hibernators which show a fluctuation in the thermal response of their membranes between the "summer-active" and "winter-torpor" condition which might involve changes in lipid composition (Aloia and Pengelley, 1979). It is unlikely that similar changes in composition could be made rapidly enough to accommodate the daily torpor and arousal of *M. schreibersii* considering the half-life for the membrane lipids of rat liver mitochondria is of the order of 1.6 days for the lipids in rapid turnover to 10 days for the slower components (Baily *et al.*, 1967).

The change in molecular ordering of membrane lipids and the increase in the E_a for succinate oxidation observed at about 23°C with mitochondria from homeothermic mammals, approximates the temperature below which metabolic dysfunction and loss of potassium and other ionic gradients occurs in these animals (Gollan *et al.*, 1957). Thus the temperature for the transition in the physical and functional properties of the membranes is just a few degrees above the T_b for survival (Precht *et al.*, 1973). For an animal with a membrane transition at 23°C in summer, lowering the temperature of the transition would be an essential prerequisite for winter torpor. *M. schreibersii* experiences torpor, during which T_b can fall to 10°C in summer (Kulzer *et al.*, 1970) as well as in winter. Thus to cope with both summer as well as winter torpor the transition temperature for the bat needs to remain below the minimum T_b. This is further biophysical evidence that many Microchiroptera are heterothermic endotherms and thus differ from both homeothermic endotherms and other mammalian hibernators.

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