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THE BIOENERGETICS, LIPID COMPOSITION AND THERMAL PROPERTIES OF LIVER MITOCHONDRIA FROM A MAMMALIAN HIBERNATOR

by

Daniel J. Pehowich

A THESIS

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ABSTRACT

In the hibernating state Columbian ground squirrels, *Spermophilus columbianus*, displayed only linear Arrhenius plots of State 3, State 4, Ca^{2+}-stimulated, and A-23187-stimulated steady-state respiration. The apparent energy of activation (*Ea*) for State 3 (ADP phosphorylation) respiration was 13.7 kcal/mol and the *Ea*'s for the other states were not significantly different from this value. Arrhenius plots were linear only in the upper temperature region for all four respiratory states in mitochondria from active animals, with an *Ea* of 9.6 kcal/mol for State 3 respiration between 22-37°C. Below 22°C rates became progressively lower than would be predicted by a strictly linear relationship with temperature.

When liver mitochondria from Richardson's ground squirrels, *Spermophilus richardsonii*, were examined during several phases of an annual hibernation cycle, it was seen that Arrhenius plots of succinate oxidation-linked H^+ ejection, Ca^{2+} uptake and oxygen consumption became non-linear at a critical temperature (*Tc*) near 12° C in spring (April, emerged from hibernation), near 21° C in summer-active (July) animals, and at 11°-13° C in warm-(20° C) or cold-(4° C) acclimated winter animals. Arrhenius plots were linear in some warm- and cold-acclimated individuals which had prepared for but had not exhibited hibernation. Linear Arrhenius plots between 4°-37° C were also typical in animals killed in hibernation (*Tb*=4° C) or 12-14 hr after arousal from hibernation (*Tb*=37° C). Mitochondrial oxygen consumption was depressed nearly 60 percent in hibernating animals compared to summer animals. These results indicate seasonal differences in the thermal behavior of mitochondrial activity commensurate with hibernation. The lowering of *Tc* in euthermic animals between summer and winter and linear plots in euthermic, temperature-acclimated animals already prepared for hibernation, and in hibernating and aroused animals, suggest these seasonal changes are endogenous in nature and are independent of ambient and body temperature variations.

The bioenergetics of mitochondrial respiration reflect the properties of the energy-transducing inner mitochondrial membrane, the chemiosmotic hypothesis of energy conservation predicting a stoichiometric coupling of proton ejection to Ca^{2+} uptake and oxygen consumption across the inner membrane. A H^+ / Ca^{2+} ratio (nmoles H^+ ejected per nmoles Ca^{2+} accumulated) near 2 was observed in mitochondria at all temperatures in

active and hibernating ground squirrels. A Ca^{2+} / O ratio (nmoles Ca^{2+} accumulated per nmole O consumed) near 4 was observed in active and hibernating animals as well. The tight coupling of Ca^{2+} uptake to H^+ ejection and oxygen consumption was maintained in hibernators whose T_b was near 4° C for several days and which showed a marked depression of mitochondrial respiratory activity. Stoichiometries remained invariant with temperature in active animals despite an apparent transition in the temperature dependence of respiratory activity below 22° C. These results indicate that hibernators are able to regulate intracellular Ca^{2+} in the hibernating state and that thermal transitions in rate functions in active animals do not necessarily result from a disruption of the integrity of the inner mitochondrial membrane.

The thermotropic behavior of the inner mitochondrial membrane was different between the summer-active and hibernating squirrels. Differential scanning calorimetry showed that the gel to liquid-crys talline phase transition temperature decreased from -2.6±0.6 °C in summer animals to -5.5 ±0.4 °C in hibernators. ^{19}F NMR analysis of the membrane showed that the order parameter, S_{mol}, was higher at all temperatures in summer animals and that the difference increased as temperature was lowered to the proximity of the phase transition. Steady-state fluorescence polarization spectroscopy using the fluoroprobe 1,6-diphenyl-1,3,5-hexatriene showed that the fluidity of the inner mitochondrial membrane was greater in hibernating animals. These results indicate that the inner mitochondrial membrane of hibernating animals is less ordered and the viscosity less (*i.e.*, more fluid) than summer animals, and that the phase transition temperature is lower in membranes from hibernators. All three physical techniques indicated that the inner mitochondrial membrane was in a liquid-crystalline state between at least 0 and 37° C in all phases of the hibernation cycle.

Fatty-acyl chain compositional analysis of the inner mitochondrial membrane total phospholipids and the major phospholipid classes (phosphatidylcholine, phosphatidylethanolamine, and cardiolipin) revealed that while the total level of fatty acid unsaturation was unchanged during hibernation, there was a shift toward increased levels of mono- and di-unsaturates, a decrease in polyunsaturates, and an increase in the ratio of (n-6) to (n-3) fatty acids in the hibernating state. This distribution of fatty acids may account for the observed thermotropic behavior of the membrane.

The general conclusions which may be derived from this study are that preparation for hibernation in ground squirrels includes an apparent alteration in the temperature dependence of mitochondrial respiration, however, only minor changes in membrane fluidity occur between the summer-active and hibernating states and may not necessarily be related to the Arrhenius characteristics of membrane activity.

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Table of Contents

Chapter — Page

ABSTRACT iv

ACKNOWLEDGEMENTS vii

LIST OF TABLES xi

LIST OF FIGURES xii

GENERAL INTRODUCTION 1

I. THE EFFECT OF TEMPERATURE ON OXIDATIVE PHOSPHORYLATION AND Ca^{2+}-STIMULATED RESPIRATION IN LIVER MITOCHONDRIA FROM ACTIVE AND HIBERNATING GROUND SQUIRRELS 4

A. Introduction 4

B. Materials and Methods 5

General 5

Animals 6

Isolation of Mitochondria 6

Assessment of Preparations 7

Measurement of Oxidative Activity 7

A-23187 10

Experiment 1.1. Effect of temperature on oxidative phosphorylation 10

Experiment 1.2. Ca^{2+}-stimulated respiration 11

Experiment 1.3. Steady-state Ca^{2+}-stimulated respiration 11

Analysis of Arrhenius Plots 11

C. Results 13

Experiment 1.1. Effect of temperature on oxidative phosphorylation 13

Experiment 1.2. Ca^{2+}-stimulated respiration 18

Experiment 1.3. Steady-state Ca^{2+}-stimulated respiration 20

D. Discussion 24

II. SEASONAL CHANGES IN LIVER MITOCHONDRIAL SUCCINATE DEHYDROGENASE ACTIVITY 26

A. Introduction 26

B. Materials and Methods 27

Animals 27

Isolation of mitochondria 27

Measurement of H^+ efflux, Ca^{2+} uptake, and O_2 consumption 28

C. Results 31

D. Discussion 37

III. STOICHIOMETRY OF H^+ EFFLUX TO RESPIRATION-DEPENDENT Ca^{2+} UPTAKE AND OXYGEN CONSUMPTION IN LIVER MITOCHONDRIA 41

A. Introduction 41

B. Materials and Methods 43

Animals 43

Isolation of mitochondria 43

Measurements of H^+ ejection, Ca^{2+} uptake and oxygen consumption 43

C. Results 45

Measurement of rates of H^+ ejection, Ca^{2+} uptake and oxygen consumption 45

Arrhenius plots of H^+ ejection, Ca^{2+} uptake and oxygen consumption 45

Stoichiometry of respiration: H^+ / Ca^{2+} and Ca^{2+} / O ratios 48

D. Discussion 50

IV. THERMOTROPIC BEHAVIOR OF INNER MITOCHONDRIAL MEMBRANE IN DIFFERENT PHASES OF HIBERNATION CYCLE 53

A. Introduction 53

B. Materials and Methods 55

Animals 55

Isolation of inner mitochondrial membrane 55

Differential scanning calorimetry 56

^{19}F Nuclear magnetic resonance spectroscopy 56

DPH Fluorescence polarization spectroscopy 56

C. Results 58

Differential scanning calorimetry 58

^{19}F Nuclear magnetic resonance spectroscopy 62

DPH Fluorescence polarization spectroscopy 62

D. Discussion 67

V. SEASONAL CHANGES IN THE FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM LIVER INNER MITOCHONDRIAL MEMBRANE 71

A. Introduction 71

B. Materials and Methods 73
Animals 73
Isolation of inner mitochondrial membrane 73
Extraction of total lipids 73
Removal of non-lipid contaminants 73
Separation of phospholipid fraction 74
Identification of phospholipid classes 74
Separation of phospholipid classes 75
Methylation of phospholipid fatty-acyl chains 75
Gas chromatography of fatty acid methyl esters 75
C. Results 77
Total phospholipids 77
Cardiolipin, phosphatidylethanolamine and phosphatidylcholine 81
D. Discussion 84
GENERAL DISCUSSION 88
REFERENCES 95

LIST OF TABLES

Table		Page
1.1	Absolute rates of oxygen consumption in mitochondria from active and hibernating ground squirrels	16
2.1	Arrhenius characteristics of respiration-coupled membrane functions during different phases of the hibernation cycle	34
2.2	Absolute rates of oxygen consumption of liver mitochondria at the temperature extremes experienced during the hibernation cycle	36
3.1	Absolute rates of mitochondrial H^+ ejection, Ca^{2+} uptake and oxygen consumption at 37° and 4° C in active and hibernating ground squirrels	49
4.1	Thermal properties of liver inner mitochondrial membrane from ground squirrels in different phases of hibernation cycle	59
5.1	Fatty-acyl chain composition of total phospholipids from liver inner mitochondrial membrane from ground squirrels in different phases of hibernation cycle	79
5.2	Influence of hibernation state on proportion of unsaturated bonds in total phospholipids from inner mitochondrial membrane	80
5.3	Fatty-acyl chain composition of major phospholipids of liver inner mitochondrial membrane from active and hibernating ground squirrels	82
5.4	Proportion of unsaturated bonds in major phospholipids from the inner mitochondrial membrane of active and hibernating ground squirrels	83

LIST OF FIGURES

Figure		Page
1.1	Oxygen consumption apparatus	8
1.2	Experiment 1.1. Arrhenius plot of ADP-stimulated succinate oxidation in active and hibernating ground squirrels	14
1.3	Experiment 1.1. Arrhenius plot of State 4 (ADP -limiting) succinate oxidation in active and hibernating ground squirrels	17
1.4	Experiment 1.2. Arrhenius plot of Ca^{2+}-stimulated succinate oxidation in active and hibernating ground squirrels	19
1.5	Experiment 1.3. Arrhenius plot of A-23187-induced steady-state Ca^{2+}-stimulated respiration in active and hibernating ground squirrels	22
2.1	Electrode assembly for the simultaneous measurement of H^+ efflux, Ca^{2+} uptake and oxygen consumption	29
2.2	Arrhenius plots of O_2 consumption as a measure of succinate dehydrogenase activity in mitochondria from ground squirrels which demonstrate a thermally induced transition in rate function	32
2.3	Arrhenius plots of O_2 consumption as a measure of succinate dehydrogenase activity in mitochondria from ground squirrels which do not show an apparent transition in enzyme function	33
3.1	Arrhenius plots of H^+ efflux, Ca^{2+} uptake and oxygen consumption in liver mitochondria from active ground squirrels	46
3.2	Arrhenius plots of H^+ efflux, Ca^{2+} uptake and oxygen consumption in liver mitochondria from hibernating ground squirrels	47
4.1	Differential scanning calorimetry thermograms of endothermic (heating) phase transitions in liver inner mitochondrial membranes from summer-active and hibernating ground squirrels	60
4.2	Differential scanning calorimetry thermograms of exothermic (cooling) phase transitions of liver inner mitochondrial membranes from summer-active and hibernating ground squirrels	61
4.3	^{19}F NMR line spectra from liver inner mitochondrial membranes from summer-active and hibernating ground squirrels	63
4.4	^{19}F NMR orientational order parameter profiles of liver inner mitochondrial membranes from summer-active and hibernating ground squirrels	64

4.5 Arrhenius plot of DPH fluorescence polarization of liver inner mitochondrial membranes from summer-active and hibernating ground squirrels 65

5.1 Representative gas chromatogram of fatty acid composition of phospholipids from liver inner mitochondrial membranes from a hibernator .. 78

GENERAL INTRODUCTION

Hibernation is a unique physiological adaptation by which some mammalian species survive low environmental temperatures and food scarcity released from the burden of high energy demands. By regulating body temperature at extremely low physiological levels, the energy conservation during hibernation is considerable and has been estimated to be as high as 80 to 90 percent (Wang, 1979). Many species of hibernators, including the Columbian ground squirrel and Richardson's ground squirrel, can maintain their body temperatures near 2-4°C for several days and yet still preserve the ability to rewarm to euthermia within a matter of hours.

Intuitively we would expect to see an increase in cold tolerance in hibernators to compensate for the marked depression in body temperature characteristic of hibernation. The exact nature of any cold tolerance which might take place may be tempered by the paradoxical periods of arousal to euthermia which are energetically demanding. At the cellular level cold tolerance has been inferred by the temperature sensitivity of activities such as mitochondrial respiration. A break in the temperature-rate function, or Arrhenius activation energy, of succinoxidase activity suggested that active ground squirrel liver mitochondria were more cold sensitive than mitochondria from hibernating animals which had linear Arrhenius plots (Raison and Lyons, 1971). The non-linear Arrhenius plots in summer animals were interpreted as being a consequence of a liquid crystalline-gel phase transition of the mitochondrial membrane lipids. Roberts *et al.* (1972) also reported a less steep, almost linear Arrhenius plot for liver mitochondria from hibernating hamsters compared to a non-linear plot in active hamsters, again suggesting a lessened temperature sensitivity in the hibernating state. The results of Raison and Lyons (1971) and Roberts *et al.* (1972) are in contrast to evidence presented by Liu *et al.* (1969) who showed no differences in Arrhenius slopes for ground squirrel liver mitochondria succinoxidase activity in active and hibernating animals. The evidence for increased cold tolerance in heart mitochondria is less convincing than in liver and to date the critical experimental results needed to clarify the situation have not been presented.

Despite the lack of any distinct increase in cold tolerance which encompasses all tissues in hibernators, a consistant trend has emerged with regard to the thermal

properties of membranes which do show evidence of increased cold tolerance. Keith *et al.* (1975) showed that the physical properties of phospholipids extracted from ground squirrel liver mitochondria exhibited the same Arrhenius characteristics as the intact organelles. This suggested a correlation between membrane functions and the physical properties of the membrane lipids. This concept has been taken further to reveal that in ground squirrel myocardial membranes a correlation is seen between membrane enzyme activity, lipid thermal properties and the lipid composition of the membrane (Raison *et al.* (1981). From this it was concluded that the increase in the proportion of unsaturation of membrane fatty acids during hibernation influenced the phase properties of the membrane, which in turn were inextricably linked to enzyme activity. Perhaps the best evidence for a lipid modification of membrane activity in hibernators comes from the elegant study of Houslay and Palmer (1978). Non-linear Arrhenius plots with Tc's near 13°C were observed in five separate liver plasma membrane enzymes from active hamsters, all of which were lowered to near 4°C in hibernators. Coincidental with the lowering of the break temperature was the lowering of the temperature at which lateral phase separations occurred in the membrane. A decrease in the temperature of lipid phase separations is consistant with increased membrane fluidity (Dipple and Houslay, 1978).

In two comprehensive reviews of the role of lipids in hibernation, Aloia (1979, 1980) noted that while in general brain tissue levels of fatty acid unsaturation increased during hibernation, the proportion of unsaturated fatty acids remained the same or declined in other tissues such as heart and liver. The overall conclusion was that without corroborative measurements of the thermotropic properties of membranes, the degree of unsaturation of membrane fatty acids did not appear to be of significance to cold tolerance during hibernation (Aloia, 1980).

The objectives of this study were to 1) establish if there is a distinct increase in the cold tolerance of ground squirrel liver mitochondria during hibernation, 2) determine that if there is a difference in cold tolerance, how and when is it manifested, 3) determine if a correlation can be established between the functional aspects of the mitochondrial membrane and its thermal properties, and 4) determine if the thermal properties of the membrane are a reflection of the fatty acid composition of the membrane. In order to answer these questions we directed our attention to the liver mitochondria and the

energy-transducing inner mitochondrial membrane in typical hibernators, the Columbian ground squirrel and Richardson's ground squirrel. Functional aspects of the mitochondria were determined by the temperature dependence of oxidative phosphorylation and succinoxidase-supported electron transport in various phases of an annual hibernation cycle. The thermal properties of the inner mitochondrial membrane from animals in the different physiological states were examined using differential scanning calorimetry (DSC), ^{19}Fluorine nuclear magnetic resonance (NMR) and fluorescence spectroscopy. The possible role of membrane lipids in the maintenance of membrane function during hibernation was determined by fatty-acyl chain compositional analysis of total phospholipids and major phospholipid classes of the inner mitochondrial membrane.

Taken together the results of this study indicate that respiratory activity in liver mitochondria from hibernating ground squirrels varies constantly with temperature, whereas there is an apparent transition in the temperature dependence of mitochondrial respiration in summer-active animals. A constant E*a* between 4-37°C in mitochondrial respiration in hibernating animals coincides with with seasonal changes in the thermotropic behaviour of the inner mitochondrial membrane; specifically, a decrease in lipid ordering and increased fluidity, and a lowering of the gel-liquid crystalline phase transition temperature. At the same time the fatty acid composition of the inner mitochondrial membrane adopts a distribution which may contribute to the thermotropic behaviour of the membrane. However, the magnitude of all the observed changes are not large and suggest that there are only minor adaptations required for the transition from the summer-active to the hibernating state.

I. THE EFFECT OF TEMPERATURE ON OXIDATIVE PHOSPHORYLATION AND Ca^{2+}-STIMULATED RESPIRATION IN LIVER MITOCHONDRIA FROM ACTIVE AND HIBERNATING GROUND SQUIRRELS

A. Introduction

Since Raison and Lyons (1971) first reported linear Arrhenius plots of succinoxidase activity in hibernating ground squirrels, and a break near 21°C in active animals, the relevance of their observations has been the focus of considerable attention. Roberts *et al.* (1972) also found differences in the thermal sensitivity of succinate respiration in hamsters, *i.e.*, a shallower Arrhenius slope during hibernation. Although these results suggest that mitochondria from hibernating animals appear to be more cold tolerant than non-hibernators and hibernators in the summer-active state, there are exceptions. For instance Liu *et al.* (1969) found no differences in mitochondrial respiration between non-hibernating and hibernating hamsters or ground squirrels.

Apart from temperature sensitivity, several studies have shown that mitochondrial respiration is depressed during hibernation (Daudova, 1968; Liu *et al.*, 1969; Shug *et al.*, 1971; Roberts *et al.*, 1972). Prior to these studies Horwitz and Nelson (1968) had demonstrated that differences in absolute rates of mitochondrial respiration between torpid and aroused bats were removed simply by washing the preparations, suggesting that any depression during hibernation was due to extramitochondrial factors. Roberts and Chaffee (1973) found no depression of succinoxidase activity in heart homogenates from hibernating hamsters. A comprehensive review on cell-homogenate and mitochondrial respiration in hibernators (Willis, 1980) emphasizes that there is no clearly defined depression of respiration which is uniquely associated with hibernators. Rather, the exhibition of cold tolerance appears to be dependent on the state of the mitochondria and the oxidative pathway used to evaluate the effect of low temperature on energy production.

While oxidative phosphorylation is a dominant role of mitochondria, electron transport can also provide the energy for ion transport across the mitochondrial membrane. In particular, the energy-transducing inner mitochondrial membrane is capable of deriving energy from the respiratory chain for Ca^{2+} uptake without the involvement of

the phosphorylation system. The early observations of Chance in 1955, that Ca^{2+} is unique in its ability to stimulate respiration temporarily and in a concentration-dependent manner, have led to the current realization that the mitochondrion is pivotal in intracellular Ca^{2+} regulation. While the stimulation of respiration is only transient upon the addition of Ca^{2+}, the presence of a divalent cation ionophore, A-23187, promotes a maximally stimulated respiratory rate as a consequence of a cyclic flux of Ca^{2+} ions across the mitochondrial membrane (Reed and Lardy 1972). The steady- state respiratory V*max* resulting from the ionophoretic action of A-23187 has been used to determine the kinetics of energy-dependent Ca^{2+} transport (Hutson *et al.*, 1976) and its temperature dependence in rat liver mitochondria (Hutson, 1977). Arrhenius plots of Ca^{2+}-stimulated mitochondrial V*max* showed a break near 23° C with an apparent increase in activation energy below this point (Hutson, 1977).

In order to clarify the concept of improved mitochondrial cold tolerance in relation to hibernation, a systematic approach appears essential. While Liu *et al.* (1969) and Raison and Lyons (1971) measured succinoxidase activity during State 3 respiration (ADP as phosphate acceptor), there is no evidence to exclude the possibility that the activity of the nucleotide translocase, which is known to be temperature sensitive (Lee and Gear, 1972) might not be limiting respiration. To circumvent this potential problem, the present study examined mitochondrial respiration under four metabolic states: State 3 (ADP as phosphate acceptor), State 4 (acceptor-limiting), Ca^{2+}-stimulated, and steady-state respiratory V*max* in the presence of Ca^{2+} and A-23187. Under these conditions it was possible to determine the temperature sensitivity of oxidative phosphorylation and the activity of the electron transport chain independently of phosphorylation.

B. Materials and Methods.

General

Double-distilled, deionized water was used to prepare all aqueous solutions. Isolation and reaction media were prepared on the day of the experiment. All glassware was washed in hot water with *Alconox* detergent, soaked in distilled water overnight and oven dried prior to use.

Animals

Adult Columbian ground squirrels, *Spermophilus columbianus*, were captured on the eastern slopes of the Rocky Mountains near Gorge Creek, Alberta. These animals were housed in the University of Alberta Biological Services facilities prior to investigation. A diet of Purina Rat Chow and vitacubes with occasional fresh lettuce was provided *ad libitum*. Animals under study were placed in environmental chambers and kept at 4° C under total darkness. A weight record of all animals was kept in order to follow the annual hibernating cycle (ground squirrels typically gain weight in preparation for hibernation (Wang, 1978)). Hibernating animals were designated as those animals which were in the proper weight cycle (high body weight) and which had experienced several bouts of hibernation as determined by daily inspection. All hibernators were killed at least three days into an established hibernating bout and had rectal temperatures of 5° C or lower. Ground squirrels designated as active (cold- acclimated) were those animals kept under the same conditions as hibernators but which showed no indication of hibernating. Food deprivation for several days failed to initiate hibernation in these animals and all were normothermic (T_b 37° C) when killed.

Isolation of Mitochondria

Animals were killed with a blow to the head. Livers were excised within 90 seconds and cooled immediately in ice-cold isolating medium. After weighing, the liver was minced with a razor blade and rinsed several times with fresh isolating medium to remove any residual blood. The tissue was then homogenized in a Potter-Elvehjem type homogenizer with a teflon pestle which had previously been turned on a lathe to a diameter which provided a clearance of 0.7 mm between it and the walls of the homogenizer. The isolation medium consisted of 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4, 1.0 mM EGTA and 0.5 mg / ml BSA. The medium was adjusted to pH 7.4 with Triethanolamine. After homogenization the homogenate was first centrifuged at 20,000 g for 10 minutes to remove any lipid released during the homogenization process which could be detrimental to mitochondrial integrity. The sediment was then resuspended and centrifuged at 600 g for 5 minutes. The resultant supernatant fluid was saved and the sediment homogenized with a tight-fitting glass pestle and centrifuged again. This step was

repeated three times for improved recovery of mitochondria. The pooled supernatants were then centrifuged at 15,000 g for 10 minutes to isolate the mitochondrial fraction. A low-speed (600 g for 15 minutes) centrifugation was then used to remove any contamination from the nuclear fraction. A final spin at 20,000 g for 15 minutes resulted in a tightly packed mitochondrial pellet which was rinsed with fresh isolating medium and resuspended to a final concentration near 50 mg protein per ml. The mitochondrial stock suspension was kept on ice until use.

Assessment of Preparations

A number of criteria were used to determine the integrity of mitochondrial preparations. The homogeneity of suspensions obtained as described above was assessed by transmission E.M. A sample was fixed in suspension with 5% glutaraldehyde in phosphate buffer at pH 7.4 and centrifuged to form a pellet. The pellet was then post-fixed with 1% osmium tetroxide in phosphate buffer, dehydrated in a graded series of ethanols and propylene oxide and embedded in Epon 812. Sections were stained with methanolic uranyl acetate and lead citrate. Another criterion used was the addition of exogenous NADH to a portion of the mitochondrial preparation while O_2 consumption was monitored. A well-prepared suspension of mitochondria should not oxidize NADH as this substrate does not pass through the mitochondrial membrane. The final criterion which must be met is demonstration of a high respiratory control ratio (State 3: State 4); only preparations yielding a ratio of 4 or greater were used.

Measurement of Oxidative Activity

Respiration was measured polarographically with a Clark type pO_2 electrode fitted to an Instrumentation Laboratories 113 Blood Gas Analyzer. The standard reaction medium consisted of 200 mM mannitol, 63 mM sucrose, 5 mM HEPES, 0.1 mM EGTA, 5 mM $MgCl_2$, 1.25 μM rotenone and 2 mg/ml BSA. Succinate (sodium salt) was used as substrate at a final concentration of 5 mM. The reaction medium was adjusted to pH 7.4 at each measurement temperature with 2 molar triethanolamine to compensate for the effect of temperature on the buffer. The pO_2 electrode was housed in a plexiglass jacket which fitted snugly into a glass reaction chamber (Fig. 1.1). A water jacket surrounding the

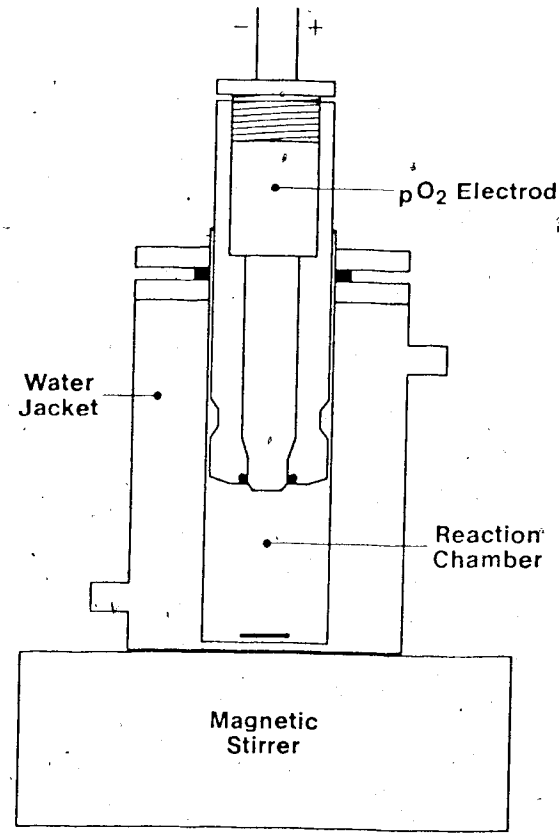


Figure 1.1. Oxygen consumption apparatus. A Clark-type pO_2 electrode is placed inside a plexiglass jacket which fits snugly into a glass reaction chamber. Mitochondria and other additions are introduced to the reaction with a Hamilton syringe. A groove along the length of the electrode jacket permits additions to the medium without allowing the entry of air bubbles. The reaction chamber is surrounded by a water jacket which can regulate the temperature to ±0.1° C.

reaction chamber maintained the desired temperature, ±0.1° C. To measure respiration, 4 ml of air-saturated medium was added to the reaction chamber, the electrode inserted and any air bubbles removed so that the reaction was essentially a closed system. Mitochondria and other additions were made to the reaction chamber with a Hamilton syringe through a groove between the pO_2 electrode and the wall of the chamber. Continuous rates of O_2 consumption were recorded on a chart recorder (Linear Instruments Corp.) which provided a permanent record of oxidative activity.

The oxygen content of the reaction medium was determined using xanthine oxidase as described by Billiar *et al.* (1970). The stoichiometry of the xanthine oxidase reaction allows for the calibration of O_2 solubility of the medium, i.e.,

$$\text{Xanthine} + H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{Uric acid} + H_2O_2.$$

Therefore, each mole of xanthine oxidized requires one mole of oxygen. In this manner if the recorder was calibrated to 100 percent O_2 content with air-saturated medium, the number of moles of xanthine needed to deplete all of the oxygen (i.e. a zero reading on the recording) is equal to the moles of oxygen dissolved in the medium. The xanthine concentration was determined by measuring the absorption at 270 nm and a molar absorbancy index of 0.10×10^4 (Kalckar, 1947).

The protein content of mitochondrial suspensions was determined by the procedure of Markwell *et al.* (1978). A 200 *ul* aliquot of each suspension was solubilized in 3 ml of an alkaline copper reagent consisting of 2.0% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate and 1% sodium dodecyl sulphate to which was added 4% $CuSO_4$ at a ratio of 100:1. Contrary to observations of Markwell *et al.* (1978), the stock alkaline reagent was not stable for more than a couple of days. The precipitate that formed on standing could be prevented if the SDS was stored separately and added immediately before solubilization of the samples, or if the reagent was heated to 100° C and cooled immediately before use. After 60 minutes of incubation at room temperature, 0.3 ml of phenol reagent (Folin-Ciocalteau) diluted 1:1 with distilled water was added to the samples and the samples mixed thoroughly. After a further 45 minute incubation, absorbance was read at 600 nm on a Pye Unicam SP 1800 spectrophotometer. A standard curve was obtained using BSA standards and a reagent blank was prepared with each set of samples. Because this method is quite sensitive (10-200 *ug*/ml), the mitochondrial samples were

usually diluted accordingly before measurement. The total free Ca^{2+} available for stimulation of respiration was determined by a modification of the method of Spare (1964). A 200 *u*l aliquot of the reaction mixture was added to 1.0 ml of 0.9% sodium deoxycholate, 0.1% NaCl and heated at 100° C for 10 minutes. After cooling, the samples were centrifuged and the supernatant fluid assayed for Ca^{2+}. A 0.5 ml aliquot of the supernatant fluid was diluted with 2.0 ml distilled water and mixed with 1.5 ml of murexide reagent. The murexide reagent was made up fresh by mixing 50 ml of stock solution (80 mg ammonium purpurate in one litre propylene glycol) with 1.5 ml of 3.75 N KOH. Ca^{2+} concentration was read at 490 nm after 5 minutes.

A-23187

As classified by Reed (1979), A-23187 belongs to the polyether-carboxylic acid family of antibiotic ionophores and is characterized by a pyrrole moiety. It is unique in that it is the only polyether ionophore specific for divalent cations. The ionophore forms an electrically neutral 2:1 antibiotic-alkali metal complex with a nitrogen atom in its structure participating in ligand binding. Ca^{2+} crosses the membrane complexed by two molecules of A-23187 which are present as the carboxylic anion and thus are able to carry 2 equivalents of H^+ back across the membrane after releasing Ca^{2+} (Pfeiffer and Lardy, 1976). A-23187, acting with the mitochondrial Ca^{2+} pump, tends to establish a futile cycle of Ca^{2+} uptake and release which uncouples the mitochondria. The efflux of Ca^{2+} out of the mitochondrion is rapid enough to significantly accelerate electron transport as long as cycling persists.

Experiment 1.1. Effect of temperature on oxidative phosphorylation

Air-saturated reaction medium was equilibrated at the temperature at which oxygen consumption was to be measured. The reaction chamber was filled with medium and the pO_2 electrode inserted, with care taken to expel all air bubbles. The reaction medium consisted of 200 mM mannitol, 63 mM sucrose, 5 mM HEPES, pH 7.4, 0.1 mM EGTA, 5 mM $MgCl_2$, 1.25 *u*M rotenone, 5 mM sodium succinate, 2 mM Tris-phosphate and 2 mg/ml BSA. After calibration of the recorder, 100 *u*l of freshly isolated mitochondrial stock was added to the reaction medium and the recording of O_2 consumption commenced. After 60

seconds, 150-200 nmoles of ADP in 2-5 *u*l medium was added. This resulted in a stimulation of O_2 consumption, which after a short linear phase returned to the initial or State 4 rate. Four or five additions of ADP usually was sufficient to exhaust the O_2 content of the medium with each addition generating a State 3-State 4 cycle. Triplicate measurements of O_2 consumption were made at each temperature.

Experiment 1.2. Ca^{2+}-stimulated respiration

Oxygen consumption was measured essentially as described above except that the reaction medium consisted of 200 mM mannitol, 63 mM sucrose, 5 mM HEPES, pH 7.4, 1.25 *u*M rotenone and 5 mM succinate only. Respiration was initiated by the addition of 100 *u*l (5 mg) of freshly isolated mitochondria to the reaction chamber. After a brief lag, respiration was linear and at this point was proceeding at State 4 rates (substrate present, ADP limiting). After 2-3 minutes, 30 nmoles Ca^{2+} per mg protein in 10 *u*l medium was added resulting in an abrupt increase in O_2 consumption. The magnitude and duration of O_2 consumption was recorded as described above.

Experiment 1.3. Steady-state Ca^{2+}-stimulated respiration

Oxygen consumption was measured as described for Ca^{2+}-stimulated respiration. Respiration was stimulated by the addition of 30 nmoles of Ca^{2+} per mg to mitochondria and then 2 nmoles of A-23187 per mg was added. After a period of linear oxygen consumption, 5 nmoles of ruthenium red per mg was added to the reaction. Ruthenium red is a potent non-competitive inhibitor of respiration-supported Ca^{2+} uptake (Reed and Bygrave, 1974).

Analysis of Arrhenius Plots

Arrhenius plots of respiratory activity were generated with a computer program which calculated the residual sum of squares for all possible combination of points fitted to two straight lines (Zar, 1974). The point at which a minimum residual sum of squares occurred was designated as a change in slope. The validity of the slope change was assessed by computing an *F* value for the variance about the lines for two distinct slopes compared to each other and to a single line through all of the points. An *F* value was also

determined for the slopes of these lines, again comparing two lines with each other and with a single line. A Student's *t* test comparing the slopes of two lines to each other and to a single line was also performed. A change in the linearity of the slope was said to occur when these tests were significant at the $P<0.005$ level or less. This method of assessing the goodness of fit of the Arrhenius plot differs from others (eg. Cook and Charnock, 1979) in that it includes a number of determinations at each experimental temperature and uses the variance between replicate measurements.

The underlying biochemical causes of non-linear Arrhenius plots of biological processes are not well understood and until a valid mechanistic interpretation of the causes of the non-linearity can be made we would have to conclude that the Arrhenius slope does not report the true reaction enthalpy (McElhaney, 1982). In this study an apparent energy of activation was only calculated for those temperature regions which were unequivocally linear as determined by our least squares program. It is apparent that only if a simple linear relationship between reaction velocity and temperature exists can we assume that the reaction geometry, activation entropy and activation enthalpy are all temperature independent (Bagnall and Wolfe, 1982).

C. Results

Experiment 1.1. Effect of temperature on oxidative phosphorylation

When added to the reaction medium, mitochondria consumed oxygen in the absence of exogenous ADP and were denoted as being in State 4 or acceptor-limiting respiration. On the addition of 150-200 nmoles of ADP, oxygen consumption increased abruptly, was linear for several seconds and then returned to the State 4 rate. Rates of State 3 and State 4 respiration were calculated from the linear segments of oxygen consumption traces.

The addition of 30 nmoles of Ca^{2+} per mg of protein to mitochondria respiring in State 4 resulted in a transient increase in oxygen consumption which returned to State 4 after several seconds. The addition of A-23187 stimulated oxygen consumption which remained linear until oxygen tension approached zero or until ruthenium red was added at which time oxygen consumption returned to State 4.

Figure 1.2 shows the effect of temperature on the rate of phosphorylation of exogenous ADP in an active ground squirrel. At 37° C *Vmax* was 67.6 ± 4.6 nmoles O_2/mg/min (mean± S.E.M.) and the rate of respiration decreased with decreasing temperature such that at 21° C the rate was 26.4 ± 1.4 nmoles O_2/mg/min and only 4.2±0.5 nmole/mg/min at 5°C (Table 1.1). When expressed as an Arrhenius plot (Fig. 1.2) the rate-temperature relationship appears to be linear in the temperature region immediately below 37°C, with an average apparent energy of activation (*Ea*) of 10.6 kcal/mole (n=5) as derived from the Arrhenius equation. As temperature decreased further, the slope of the Arrhenius plot became non-linear and was steeper in the temperature region of 23-5°C. That the rate of succinate oxidation was occurring at *Vmax* was confirmed by the addition of higher concentrations of ADP which failed to increase the rate but merely prolonged the duration of oxidation. The addition of an uncoupler, 2,4-dinitrophenol, did increase the rate of oxidation to a maximum of 114.9 ± 5.0 nmoles O_2/mg/min at 37° C. This would indicate that the electron transport chain is not rate-limiting for the phosphorylation of ADP.

When the phosphorylation of exogenous ADP was measured in hibernating ground squirrels, Arrhenius plots were substantially different from those of active animals. The

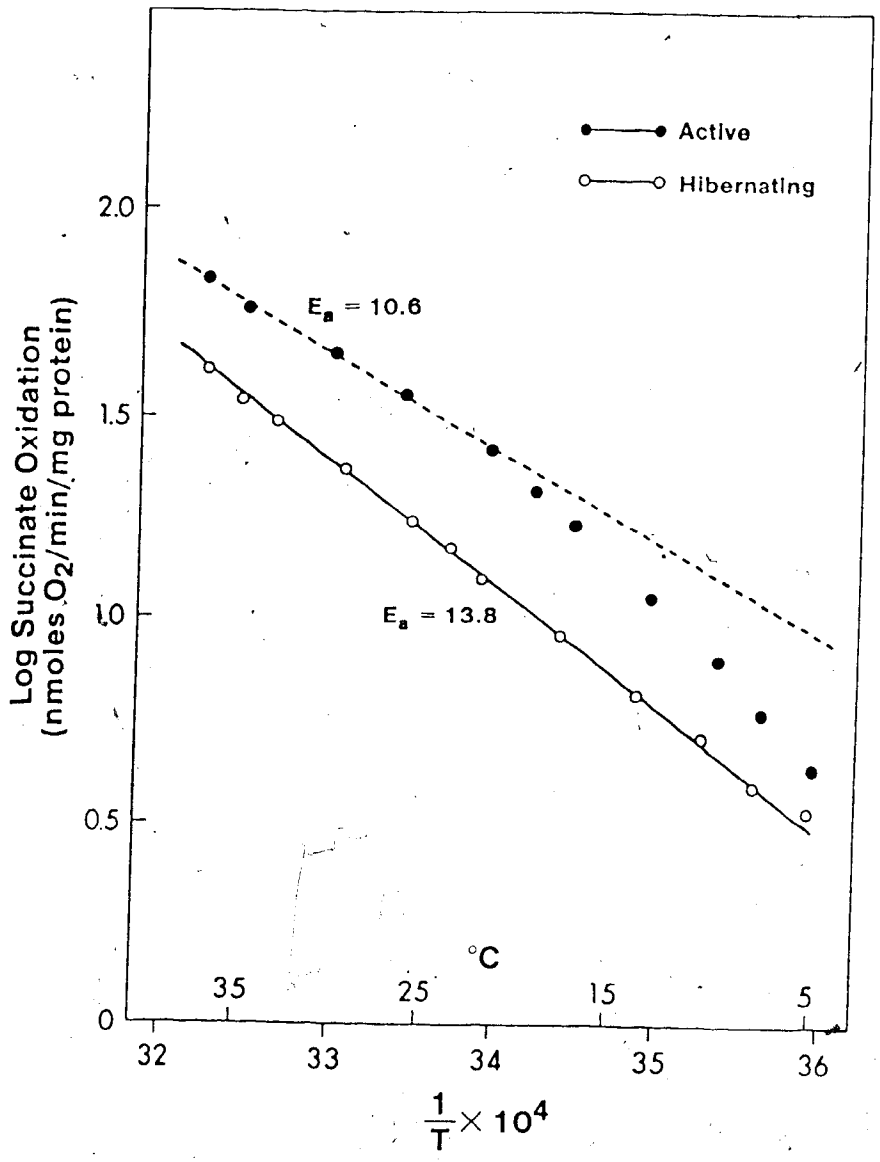


Figure 1.2. Experiment 1.1. Arrhenius plot of ADP-stimulated succinate oxidation in liver mitochondria from active and hibernating Columbian ground squirrels. Ea's are the mean of six individuals.

most conspicuous difference was the absence of any discontinuity or "break" in the Arrhenius plot, *i.e.*, th slopes were linear between 5 and 37°C (Fig. 1.2). The average experimentally determined Ea was 13.8 kcal / mol over that temperature range (n=6).

The absolute rates of phosphorylation of ADP, as determined by succinate oxidation, were lower in hibernating animals than in active animals. At 37° C V*max* was 41.9 ± 2.8 nmoles O_2 / mg / min, nearly 40% less than the corresponding temperature in active animals ($P<0.01$). In hibernating animals the rate at 21° C was less than half that of active animals (12.2 ± 1.1 *vs* 26.4 ± 1.4 nmoles O_2 / mg / min), however, at 5° C the difference between the two states was not as large (3.0 ± 0.4 *vs* 4.2 ± 0.5 nmoles O_2 / mg / min)(Table 1.1)., but was significant.

The State 4 rate of respiration in active and hibernating ground squirrels had nearly identical Arrhenius characteristics to those of State 3 respiration. The Arrhenius plot of oxygen consumption in active animals became non-linear below about 22°C, the average Ea to that point was 9.7 kcal / mol (Fig. 1.3). In comparison, there was only a single apparent activation energy applicable to rates of respiratory activity in hibernating animals. Any segment of a regression between 5 and 37° C did not yield an individual Ea significantly different from the overall value. The Ea's in the upper linear region in active animals were significantly different from the average Ea in hibernating animals (9.7 *vs* 13.9 kcal / mole).

The magnitude of the increase in respiration that occurs during the phosphorylation of ADP can be seen when comparing State 4 to ADP-stimulated (State 3) rates of succinate oxidation (Table 1.1). The ratio of the two rates, the respiratory control ratio (RCR), is a measure of how tightly coupled oxidation is to phosphorylation and is thus an indicator of the energy state of the mitochondria. The RCR between State 3 and State 4 respiration during hibernation was greater than 5 (e.g. 16.4 ± 3.8 *vs* 2.8 ± 0.4 kcal / mole at 25° C). This would suggest that the mitochondria of the hibernators were tightly coupled and in a state of low energy conservation, and indeed the freshly isolated mitochondria typically exhibited State 4 rates of oxidation less than 0.5 nmole O_2 / mg of protein / min at 5° C.

Table 1.1. Absolute rates of oxygen consumption in liver mitochondria from active and hibernating Columbian ground squirrels.

Respiratory State		Oxygen Consumed (nmoles/mg/min)		
		37 °C	21 °C	5 °C
ADP-stimulated	Active	67.6±4.6	26.4±1.4	4.2±0.5
	Hibernating	41.7±2.8	12.2±1.1	3.0±0.4
State 4	Active	11.6±1.5	5.0±0.6	0.7±0.09
	Hibernating	6.9±1.1	2.1±0.4	0.5±0.09
Ca^{2+}-stimulated	Active	26.9±1.9	11.2±1.5	2.0±0.3
	Hibernating	15.9±1.4	4.9±0.6	1.3±0.2
Steady state Ca^{2+}-stimulated (A-23187)	Active	95.5±5.5	38.5±1.6	5.9±0.3
	Hibernating	57.5±2.4	17.2±0.6	4.5±0.3

Values are mean ± S.E.M.; (n) = 6 for each group.

The rates of oxygen consumption were different at all temperatures in hibernating animals as determined by Student's t-test ($P<0.01$).

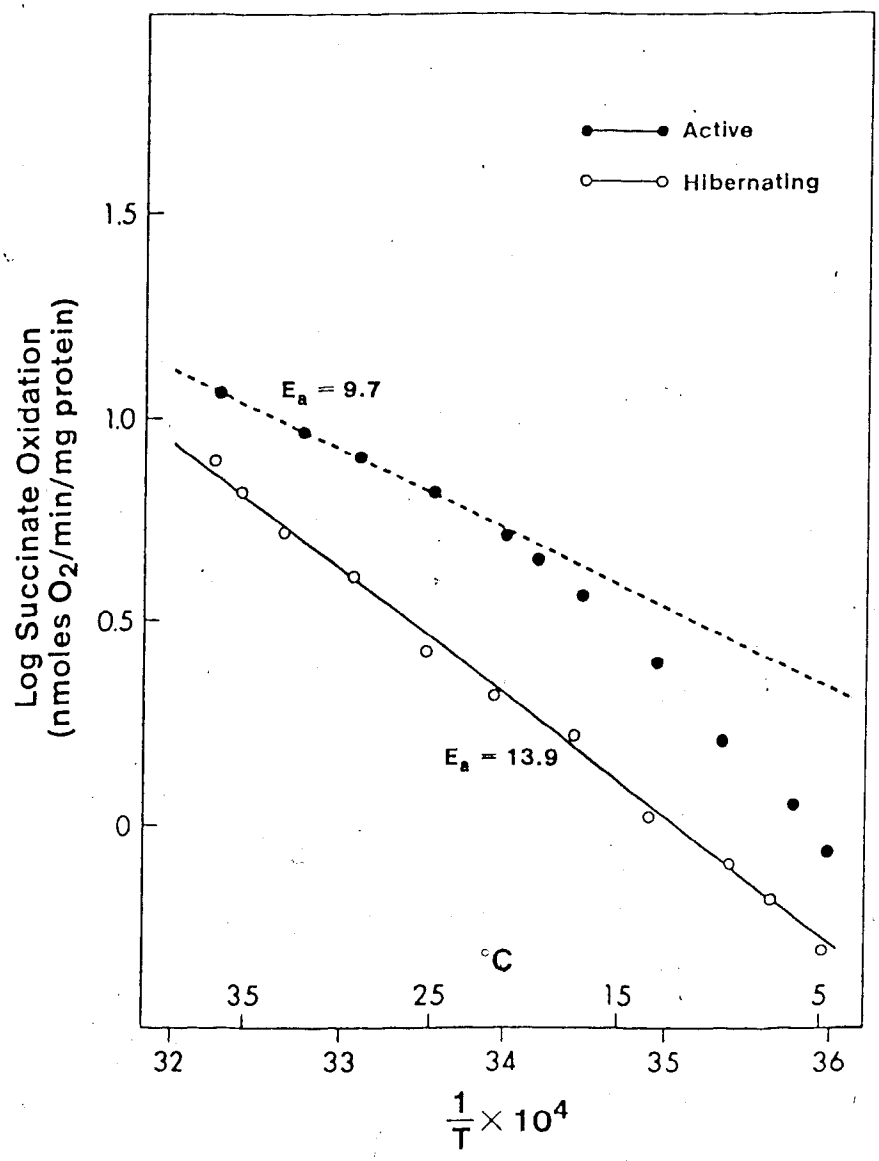


Figure 1.3. Experiment 1.1. Arrhenius plot of State 4 (ADP limiting) succinate oxidation in liver mitochondria from active and hibernating Columbian ground squirrels. Ea's are means of six individuals.

Experiment 1.2. Ca^{2+}-stimulated respiration

The addition of 30 nmoles per mg of Ca^{2+} stimulated respiration in every preparation and at every temperature. Measurable Ca^{2+}-stimulated respiration in the absence of exogenous ADP, phosphate, or Mg^{2+} in the medium demonstrated that these were not required for the uptake of Ca^{2+} by mitochondria, either in the active or hibernating state, and that respiration-linked Ca^{2+} uptake was possible at all temperatures measured (5-37° C). However, endogenous levels of these substances were not determined. The return to the initial respiratory rate is good evidence that at these concentrations of Ca^{2+} the mitochondria were undamaged and retained complete respiratory control.

When the rates of Ca^{2+}-stimulated respiration are displayed as an Arrhenius plot (Fig. 1.4), the relationship of activity to temperature was strikingly similar to State 4 and ADP-stimulated respiration. In active animals the average *Ea* was 10± 1.4 kcal/mol as the assay temperature was lowered from 37°C. The slope of the Arrhenius plot became non-linear and progressively steeper between 20 and 5°C. Arrhenius plots of hibernating animals were distinctly linear between 37-5°C, however, with an average *Ea* of 13.6± 1.1 kcal/mol. As with State 4 and ADP-stimulated respiration the average *Ea* of the linear region of Arrhenius plots of active animals was significantly different from the *Ea* for hibernators.

Of particular interest is the observation that the rate of oxygen consumption upon the addition of Ca^{2+} is intermediate between those of State 4 and ADP-stimulated activity. This suggests that while Ca^{2+} uptake is driven by respiration under the conditions used, the uptake is not limited by the electron transport chain, nor by the adenine nucleotide translocase (the higher rates of ADP-stimulated *Vmax* and uncoupler-stimulated *Vmax* indicate a much greater respiratory capacity than reached by Ca^{2+} uptake). The addition of higher concentrations of Ca^{2+} failed to increase the rate of respiration but did prolong its duration. At concentrations of Ca^{2+} greater than 100 nmoles/mg, respiration failed to return to initial rates indicating some uncoupling of respiration, either by damage to the mitochondrial membrane or the inability of the mitochondria to accumulate and retain large amounts of the ion.

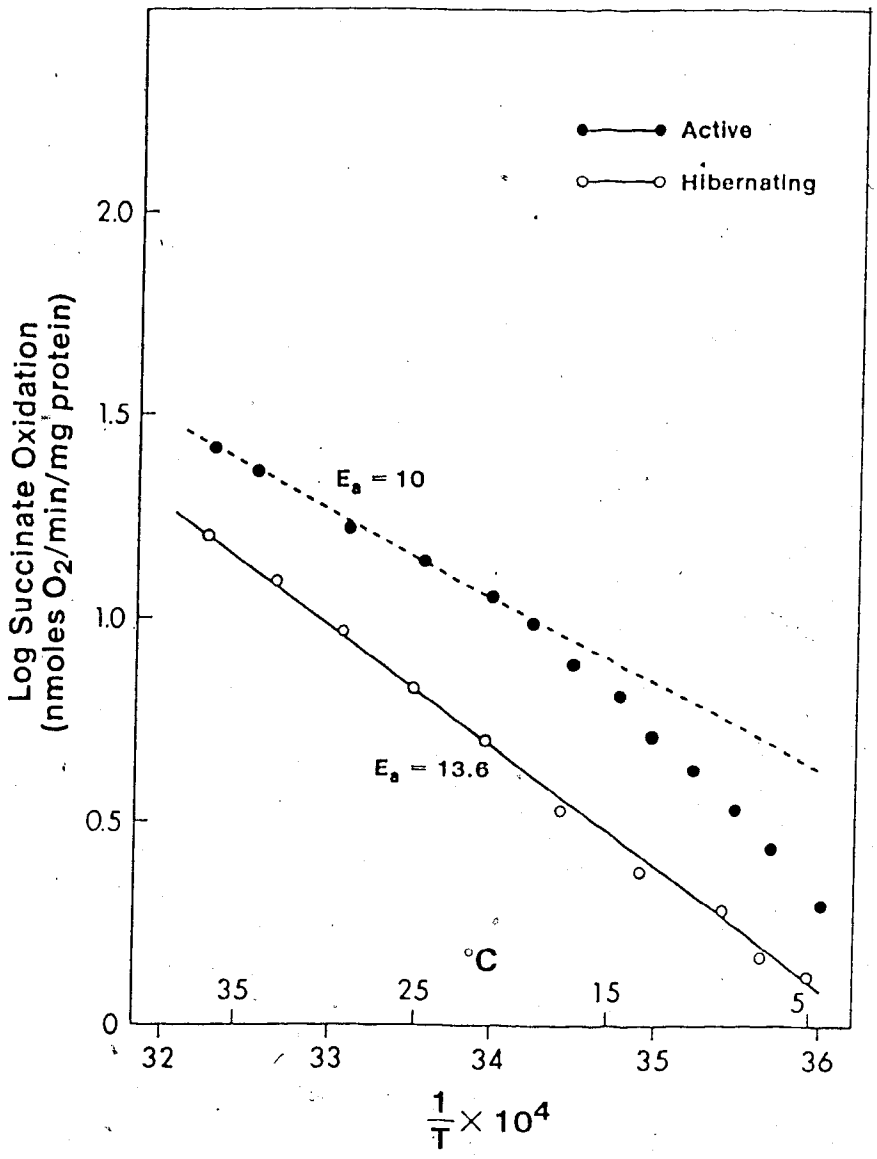


Figure 1.4. Experiment 1.2. Arrhenius plot of Ca^{2+}-stimulated succinate oxidation in liver mitochondria from active and hibernating Columbian ground squirrels. Ea's are the mean of six individuals.

Experiment 1.3. Steady-state Ca^{2+}-stimulated respiration

The addition of A-23187 to mitochondria previously loaded with 30 nmoles / mg of Ca^{2+} initiated a steady-state rate of oxygen consumption. At higher temperatures (>25° C) the increase in respiration upon the addition of the ionophore to respiring mitochondria was abrupt and linear, until the oxygen tension of the reaction medium approached zero. At lower temperatures the increase in respiration promoted by the ionophore was somewhat slower, perhaps reflecting a temperature-dependent transport of A-23187 into the mitochondrial membrane. There did not appear to be any differences in the aspects of steady-state respiration between active and hibernating animals with regard to its rate of initiation by the ionophore A-23187.

That Ca^{2+} cycling was stimulating respiration at *Vmax* in the presence of A-23187 was confirmed when the addition of more Ca^{2+} (up to 100 nmoles / mg) or more A-23187 (up to 10 nmoles / mg) failed to increase the rate of respiration. Furthermore, the addition of DNP did not increase respiration significantly at 37° C. This would indicate that substrate oxidation or electron transport was not rate-limiting for Ca^{2+}-stimulated respiration. When 5 nmoles of ruthenium red per mg was added to mitochondria respiring at *Vmax*, respiration abruptly returned to the original State 4 rates and remained constant until O_2 exhaustion . As ruthenium red is a potent inhibitor of Ca^{2+} uptake, this confirmed that Ca^{2+} uptake was the cause of the increased activity of the respiratory chain. There was no discernible difference in the inhibition of Ca^{2+} uptake by ruthenium red between the active and hibernating states, nor was there any temperature effect, i.e., Ca^{2+}-stimulated respiration was always inhibited nearly 100 percent at all temperatures. Oxygen uptake could not be stimulated by A-23187 in the presence of 1.0 mM EGTA, indicating again that Ca flux is necessary to stimulate respiration.

After the addition of ruthenium red and after the preparation had become anaerobic, the total potentially free Ca^{2+} available was assessed using murexide. Because Ca^{2+} re-uptake had been blocked, all free Ca^{2+} should have been released to the medium as a consequence of the activity of A-23187 and an anaerobic state (respiration is required to retain accumulated Ca^{2+}). The amount of free Ca^{2+} available for the stimulation of respiration ranged between 50 and 75 nmoles / mg. The addition of A-23187 to respiring mitochondria without prior loading of Ca^{2+} also enhanced respiration but not to *Vmax*.

Vmax was only attained after a subsequent addition of 30 nmoles Ca^{2+} / mg. The state of the animal also had an effect on A-23187-stimulated respiration in that the rate of ionophore-stimulated respiration in the absence of exogenous Ca^{2+} was greater in hibernators than in active animals.

The effect of temperature on the succinate-supported, Ca^{2+}-stimulated *Vmax* is illustrated in Fig. 1.5. Again, there was little difference in Arrhenius characteristics when compared to State 3, State 4, or Ca^{2+}-stimulated respiration. Plots of oxygen consumption in mitochondria from active squirrels were initially linear with an average *Ea* of 10.3±1.6 kcal / mol, but became non-linear as temperature decreased. Plots of oxygen consumption for hibernating animals were linear between 5 and 37°C with an average *Ea* of 13.7±0.93 kcal / mole.

When ADP-stimulated is compared with steady-state Ca^{2+}-stimulated O_2 consumption (Table 1.1), Ca^{2+} stimulated respiration rates were more than 40 percent higher than were possible with ADP over the entire temperature range (5-37° C). Thus, Ca^{2+} uptake may proceed during hibernation independent of the phosphorylation of ADP and at a rate higher than if its energy were derived from the hydrolysis of ATP.

When rates of Ca^{2+}-stimulated are compared with A-23187-stimulated respiration it is evident that without cycling of the ion across the mitochondrial membrane, Ca^{2+} does not stimulate respiration maximally. Oxygen consumption during the A-23187-induced cycling of Ca^{2+} is 3.5 fold greater than during Ca^{2+} uptake alone (Table 1.1). That this relationship holds true during hibernation is particularly important to note (e.g., 6.6 ± 0.6 *vs* 23.7 ± 1.4 nmoles O_2 / mg / min at 25° C). This demonstrates that in the absence of an ionophoretic agent, Ca^{2+} cannot stimulate respiration as effectively as ADP.

The wide range of oxygen consumption rates mitochondria can generate is revealed when steady-state Ca^{2+}-stimulated is compared with State 4 respiration (Table 1.1). At 5° C (near the physiological temperatures experienced during deep hibernation), the mitochondria are probably tightly coupled and consume a mere 0.5 nmoles of O_2 per mg of mitochondrial protein per minute. At normothermia in the active animal, as much as 100 nmoles of O_2 (200-fold increase) can be consumed under maximal stimulation of respiration by cycling Ca^{2+}. In comparing only State 4 rates in active and hibernating animals (Table 1.1), a 95 percent conservation of energy appears possible (11.6 *vs* 0.5

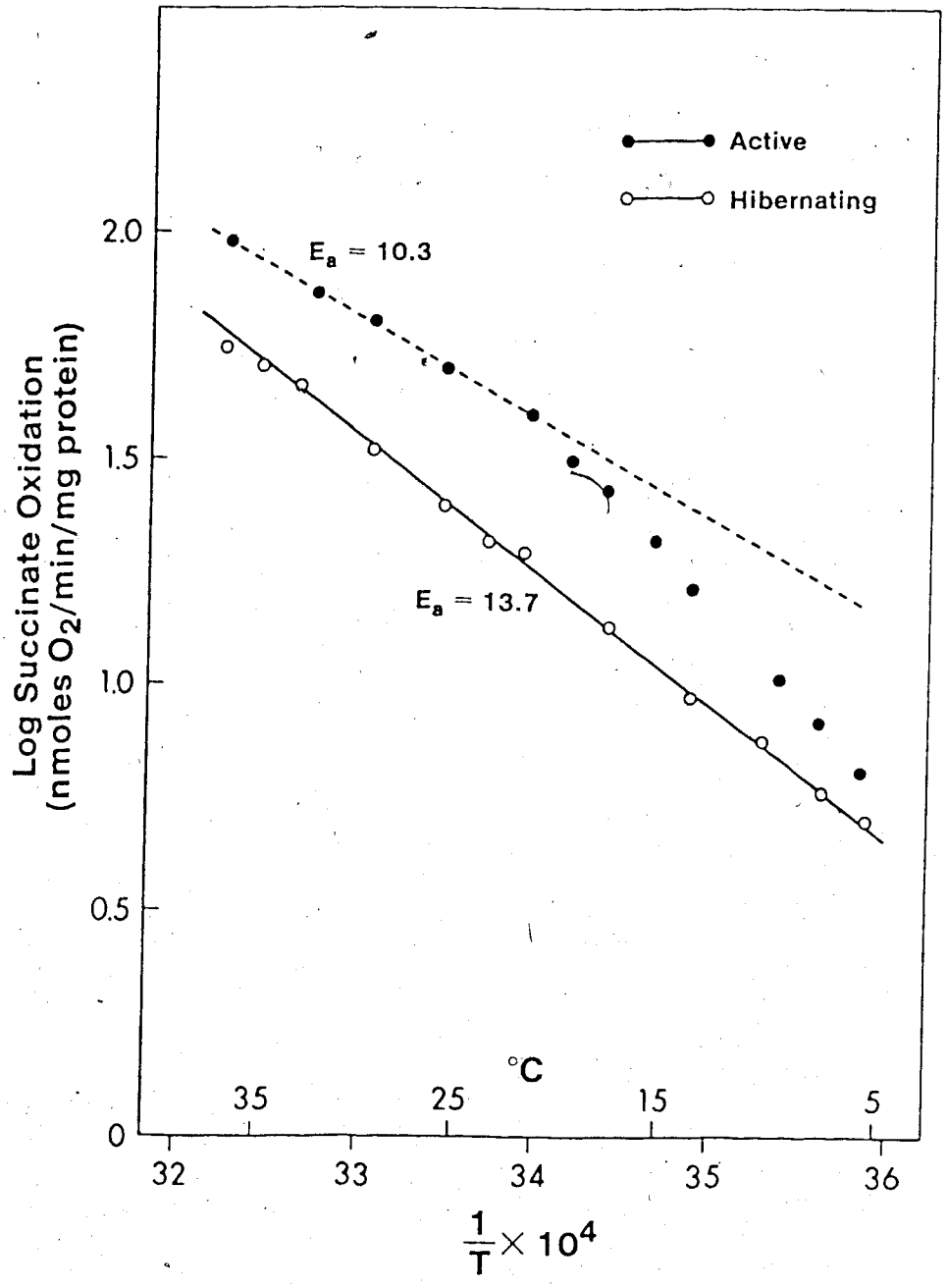


Figure 1.5. Experiment 1.3. Arrhenius plot of A-23187-induced steady-state respiration in liver mitochondria from active and hibernating Columbian ground squirrels. Ea's are the mean of six individuals.

nmoles / mg / min). It is important to stress, however, that the absolute rates of oxygen consumption obtained under these conditions are specific for the substrate and cofactors added and may not reflect the ultimate respiratory capabilities of the tissue *in situ*.

D. Discussion

This study has demonstrated a difference in the temperature sensitivity and rate of mitochondrial respiration between active and hibernating Columbian ground squirrels. Mitochondria isolated with procedures which yield tightly-coupled respiration (high State 3: State 4 ratios) showed nearly identical Arrhenius characteristics during four separate metabolic states: State 3, State 4, Ca^{2+}-stimulated, and A-23187-stimulated steady-state respiration.

Respiration in the presence of ADP as phosphate acceptor showed a strictly linear Arrhenius relationship in hibernating animals, however, plots of respiration in active animals were only linear in the upper temperature range and became non-linear below 20-23°C. These results are similar to the observations of Raison and Lyons (1971) but appear to contradict the observation of non-linear Arrhenius plots in hibernating 13-lined ground squirrels (Liu *et al.*, 1969). A non-linear Arrhenius plot in State 4 respiration in active animals suggests that the nucleotide translocase is not a rate limiting factor as State 4 respiration does not normally require its activity, yet an apparent transition in the temperature dependence of respiration is still seen. An inhibition of the translocase has been shown to occur near 12 °C in rat liver mitochondria (Lee and Gear, 1974), however, there is evidence that this inhibition is abolished in hypothyroid rats, a condition in which the total unsaturation of membrane fatty acids increases. To date there has been no examination of nucleotide translocase temperature sensitivity in mitochondria from hibernators and it is possible that it does not hinder oxidative phosphorylation at low temperature, even in active animals.

The accumulation of Ca^{2+} by mitochondria has been well studied in non-hibernating species (see Saris and Akerman, 1980 for review) and based on steady-state kinetics it is thought that the rate limiting step for Ca^{2+} uptake is respiratory chain electron transport (Hutson, 1977). The non-linear Arrhenius plots of Ca^{2+}-stimulated respiration in active squirrels indicates that there is a thermally induced transition in the rate function of the electron transport chain as temperature is lowered. This conclusion is based on the supposition that only the rate-limiting step of a complex biological reaction will yield valid Arrhenius "breaks" (Silvius and McElhaney, 1980). In the absence of an ionophore, Ca^{2+} uptake was limited to State 4 respiration and did not stimulate respiration maximally. When

A-23187 was present, maximal stimulation of respiration occurred as the result of Ca^{2+} cycling across the inner membrane. The A-23187-stimulated steady state V*max* was tightly linked to the maximal rate of electron transport and probably reflected the rate of proton translocation (Heaton and Nicholls, 1976). Again, this rate-limiting step yielded a non-linear Arrhenius plot indicating a thermally induced transition in the rate function. Hibernators, on the other hand, showed only linear Arrhenius plots for Ca^{2+} and A-23187-stimulated steady-state respiration and from this we can infer that there has been a change in the temperature sensitivity of the electron transport system in preparation for hibernation. Because the rate-temperature relationship becomes non-linear in active animals, the simple Arrhenius equation cannot be used to calculate an apparent energy of activation below the point of slope change.

Perhaps one of the reasons for a divergence in the evidence for and against increased cold tolerance in hibernators is the choice of oxidative pathway examined by different investigators. In this study only the pathway from succinate to oxygen was utilized as any spurious contributions by other components of the citric acid cycle were blocked by rotenone. Succinate dominates over NAD-linked substrates in the stimulation of oxidative phosphorylation; however, it has been shown that succinate is an even more powerful effector of Ca^{2+} uptake by mitochondria (Kondrashova *et al.*, 1982). The kinetic predominance of succinate over NAD-linked substrates such as *B*-hydroxybutyrate can be as high as 30 to 40 percent, and for this reason the choice of succinate as substrate appears to be appropriate for the evaluation of respiratory activity.

The lower rates of respiration seen in hibernators in this study does little to clarify some earlier findings which suggested that there is no difference in respiratory activity between active and hibernating ground squirrels (eg. Daudova, 1968; Liu *et al.*, 1969; Roberts *et al.*, 1972). All mitochondrial preparations used in this study were washed with medium containing EGTA, a chelating agent with a high affinity for Ca^{2+}. Mg^{2+} was not removed from preparations and there is good evidence that Mg^{2+} can inhibit respiration in cold-acclimated rats and hamsters (Rochelle *et al.*, 1978). On the other hand succinate oxidation is not impaired by Mg^{2+} depletion (Reed and Lardy, 1972). Because Mg^{2+} is antagonistic to Ca^{2+} uptake (Hunter *et al.*, 1976), an examination of the role of these two ions in hibernation seems warranted.

II. SEASONAL CHANGES IN LIVER MITOCHONDRIAL SUCCINATE DEHYDROGENASE ACTIVITY

A. Introduction

An investigation of the temperature dependence of some key enzymic reactions and rate processes is necessary if we are to resolve the differences seen in the respiratory activity of mitochondria from hibernating and non-hibernating animals. The relationship between membrane rate processes and temperature is frequently assessed with the aid of Arrhenius plots, a non-linear rate-temperature function being taken to indicate a transition has occurred at a characteristic "break" or critical temperature (*Tc*, the intercept of two slopes). A plot yielding only a single slope reflects the lack of a transition over the temperature range examined. An increased slope below the *Tc* represents an increase in the apparent energy of activation (*Ea*), indicating that the enzymic process becomes energetically less feasible. Using these criteria, thermal transitions have been observed in succinate oxidase activity in liver mitochondria of summer-active golden-mantled ground squirrels while in hibernating individuals no transition could be detected between 5-37° C (Raison and Lyons, 1971). In the Columbian ground squirrel, ADP- and Ca^{2+}-stimulated respiration in liver mitochondria were non-linear in active animals but linear during hibernation (Pehowich and Wang, 1981). Since the succinate oxidase-driven respiration is dependent upon the activity of membrane-bound enzymes of the respiratory chain, these observed differences in Arrhenius characteristics suggested an alteration in the thermal response of these enzymes in conjunction with hibernation. There is no experimental data, however, to indicate whether such an alteration in thermal behaviour is a prerequisite for hibernation or a consequence of the depressed *Tb* characteristic of the hibernating state.

In view of this we have examined the temporal aspects of mitochondrial respiratory activity during different phases of the annual hibernation cycle. Liver mitochondrial proton efflux, Ca^{2+} uptake and O_2 consumption were measured in spring and summer animals, warm- and cold-acclimated fall and winter animals, and in hibernating and aroused animals. Using this approach we may determine how preparation for hibernation is manifested in biochemical alterations of mitochondrial function.

B. Materials and Methods

Animals

Richardson's ground squirrels, *Spermophilus richardsonii*, were captured near Edmonton and provided with lab chow and water *ad libitum* with an occasional supplement of sunflower seeds. Except for the spring (April) and summer (July) captures, which were killed within two weeks, all animals were weighed weekly in order to monitor endogenous changes in the physiological state of the animal's circannual cycle. A rapid rise in body weight followed by a plateau usually signals the commencement of the hibernating phase, whereas a stable, low body weight indicates the animal is in the non-hibernating phase. Those animals designated as warm-acclimated were kept at 18-20° C and 12L:12D photoperiod and cold-acclimated animals at 4° C and 2L:22D photoperiod for a minimum of 4 weeks and were assayed on Oct.-Nov.. None of these animals had experienced torpor. Hibernating animals were killed at least three days into an established hibernation bout at a body temperature (T_b) of 5 ± 1° C. Aroused animals were those which had been hibernating several days and rewarmed spontaneously or were disturbed by handling. These animals were killed 12-14 hr. after the initiation of arousal and were euthermic (T_b=37° C).

Isolation of mitochondria

Liver mitochondria were prepared in ice-cold isolation medium consisting of 0.25 M sucrose, 0.5 mM EGTA, 0.5% BSA, and 3.0 mM NaHEPES, pH 7.4. The liver was first homogenized with a loose-fitting Teflon pestle and the homogenate centrifuged at 1,100 g. for 3 min.. The resultant supernatant was saved and the pellet homogenized in a Potter-Elvehjem tissue grinder with a tight-fitting glass pestle. The homogenate was centrifuged at 1,100 g. for 3 min., the supernatant saved and the process repeated twice more. The pooled supernatants were centrifuged at 12,000 g. for 15 min. and the resultant pellet resuspended and centrifuged at 20,000 g. for 10 min. The final mitochondrial pellet was washed twice and resuspended in 0.25 M sucrose, 2.0 mM NaHEPES, pH 7.4. Protein was determined after solubilization in sodium dodecyl sulphate (Markwell *et al.*, 1978). When examined with E.M., mitochondrial suspensions prepared in

this manner were seen to be relatively free of other organelles. The mitochondria were impervious to added NADH and tightly coupled as indicated by high acceptor control ratios.

Measurement of H^+ efflux, Ca^{2+} uptake, and O_2 consumption

Reactions were carried out in a thermostated glass chamber in which a thermocouple had been embedded to accurately measure the incubation temperature. Proton movements were followed with a high impedance combination pH electrode (Ingold Inc. model 6028-02) coupled to a Radiometer PHM64 meter. Ca^{2+} uptake was measured with a Radiometer F2112 Selectrode connected to a Corning 135 pH/Ion meter; the output was linearized with an antilog converter employing a Burr-Brown 4127KG module. Oxygen consumption was measured polarographically with a Transidyne model 731 Clark-type electrode covered with a 15 *u*M membrane. All three electrodes were sealed in a Plexiglass plunger which fit tightly into the reaction chamber (Fig. 2.1). The rates of H^+ and Ca^{2+} movement were recorded simultaneously on a dual- channel Linear recorder, the sensitivity being increased by supplying an offset voltage between the pH and ion meters and the recorder. Oxygen consumption was recorded on a single channel Linear recorder. Experiments were begun with the addition of 0.1 ml (10 mg mitochondrial protein) of stock suspension to 4.9 ml of reaction medium consisting of 120 mM sucrose, 60 mM KCl, 2 mM NaHEPES, 2 nmoles/mg rotenone, and 1 *u*g/mg oligomycin. The reaction medium was in equilibration with air and the pH adjusted to 7.4 at each of the incubation temperatures (4-37° C). Calcium chloride (10mM) was added to the reaction vessel in 4 or 5 precisely measured injections (10 *u*l each) to calibrate the Ca^{2+} electrode to a final concentration of 60 nmoles Ca^{2+}/mg mitochondrial protein. The pH electrode was calibrated with several additions of 0.1 N HCl or 0.1 N KOH. The solubility of oxygen in the reaction medium was calculated for each temperature used according to Billiar *et al* (1970). Any endogenous substrates which might be present were depleted by an initial 2 min incubation by which time the chart traces indicated the mitochondria to be completely de-energized. Respiration and ion transport were initiated by the addition of 2.0 mM succinate (final concentration) and the initial linear rates of H^+ efflux, Ca^{2+} uptake and O_2 consumption were measured. It was assumed that these represented the maximal rates as

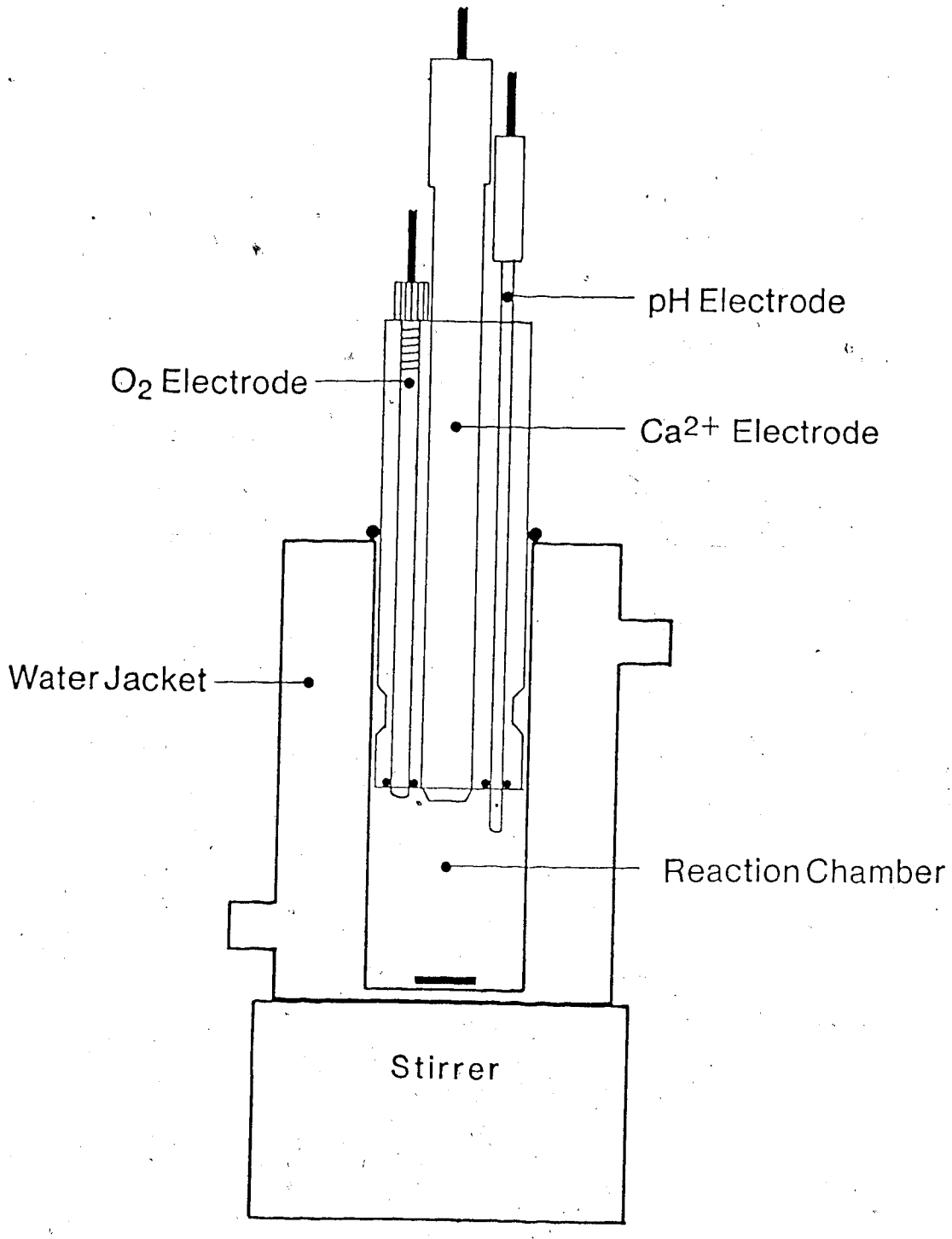


Figure 2.1. Electrode assembly for the simultaneous measurement of H^+ efflux, Ca^{2+} uptake and oxygen consumption.

succinate dehydrogenase has been shown to be fully activated by succinate (Zeylemaker, *et al.*, 1971; Vinogradov, *et al.*, 1977) and Ca^{2+} (Ezawa and Ogata, 1979).

C. Results

Arrhenius plots of liver mitochondrial O_2 consumption for the six groups of animals examined are shown in Figs. 2.2 and 2.3. In summer-active ground squirrels Arrhenius plots were initially linear as temperature was lowered from 37°C, but became non-linear near a *Tc* of 21°C. An average apparent energy of activation (*Ea*) of 9.2 kcal / mole was calculated for the linear region. The Arrhenius characteristics of H^+ and Ca^{2+} movements were nearly identical to those of O_2 consumption (Table 2.1). Ground squirrels acclimated to 20° C in fall / winter could be separated into two distinct groups (Table 2.1). One group (WA(I), n=7) exhibited linear Arrhenius plots between 37°C and a *Tc* near 13°C with an average *Ea* of 9.6 kcal / mole calculated above *Tc*. The *Ea* s for Ca^{2+} uptake and H^+ efflux were not significantly different from those values found for O_2 consumption (Table 2.1). In the second group (WA(II), n=5) Arrhenius plots were linear between 4-37°C, the *Ea*'s for the rate processes being greater than those in the WA(I) group. Body weight records of the WA(II) group showed them all to be in the steep ascending or plateau phase of a weight gain pattern in contrast to the low or declining pattern of group WA(I) animals. Ground squirrels acclimated to 4° C during the fall / winter could also be clearly divided into two groups (Table 2.1). One group (CA(I), n=4) yielded Arrhenius plots that were only linear to near 11° C, while the other group (CA(II), n=4) displayed only a single *Ea* down to 4° C (Fig. 2.2). Again the *Ea*'s of CA(II) were greater than the average *Ea* calculated for the linear temperature region in CA(I) animals (Table 2.1). Similar to that observed in the WA(II) groups, the body weight status of the CA(II) group also showed an increase or plateau pattern whereas those animals in CA(I) were either in a declining phase or showed no weight gain at all.

Animals in deep torpor (*Tb*=5° C) were linear with respect to Arrhenius plots of O_2 (Fig. 2.2), Ca^{2+}, and H^+ activity (Table 2.1). Only a single activation energy (15.2 kcal / mole) was discernible over 4-37° C for all three processes. These single *Ea*'s were higher than the average *Ea* calculated for all of the non-hibernating animals showing non-linear Arrhenius plots ($P<0.001$). Arrhenius plots of animals which had aroused from hibernation were similar to those in torpor, even though these animals were euthermic (*Tb*=37° C) at time of sampling. While the *Ea*'s for all rate processes were significantly different ($P<0.005$) from the *Ea* calculated in spring, summer, warm-, and cold-acclimated animals,

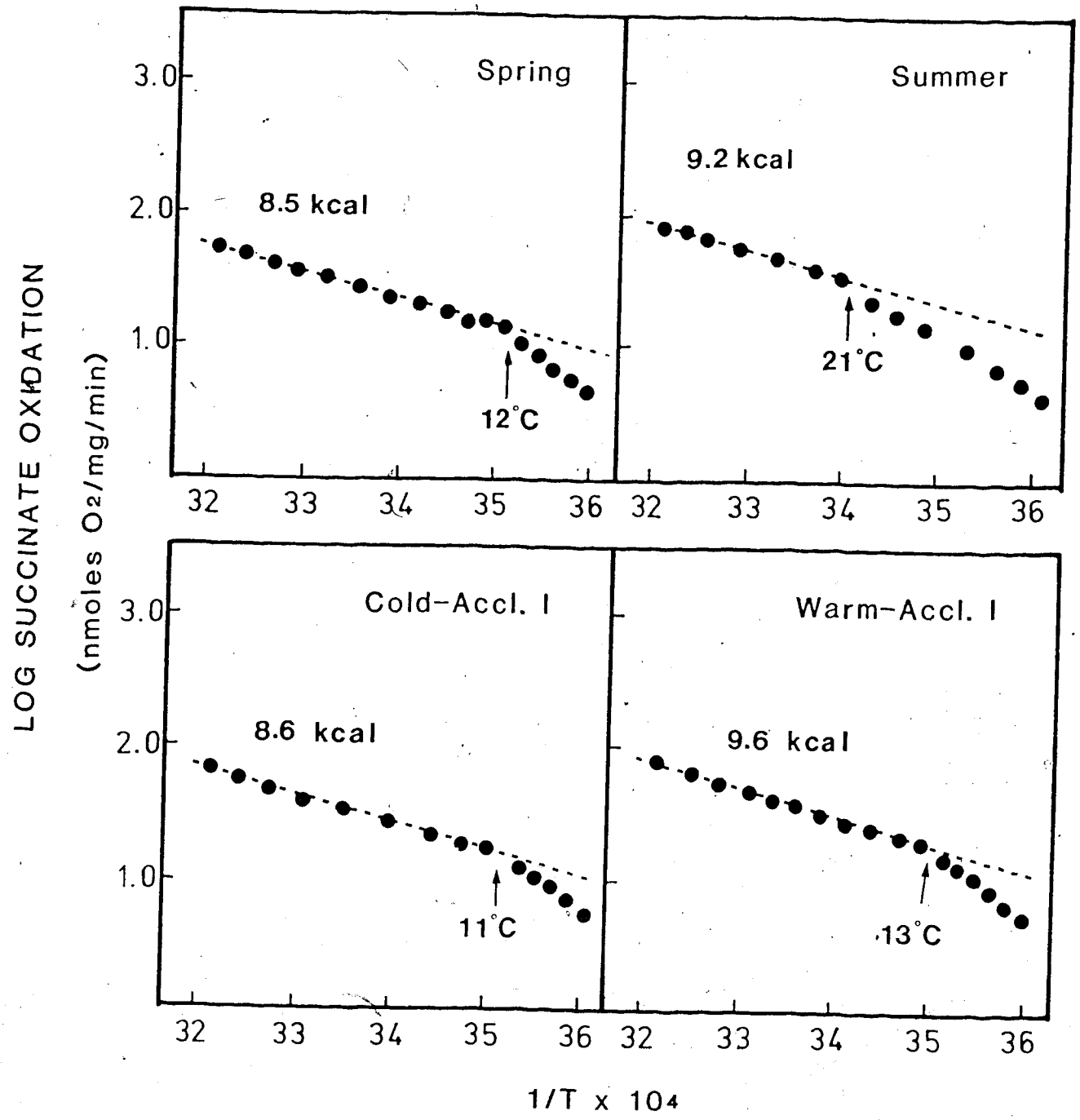


Figure 2.2. Arrhenius plots of O_2 consumption as a measure of succinate dehydrogenase activity in mitochondria from Richardson's ground squirrels which demonstrate a thermally induced transition in rate function. Each point represents the mean for several individuals (see Table 2.1 for (n)). The number beside each line is the Arrhenius activation energy (*Ea*) in kcal/mole.

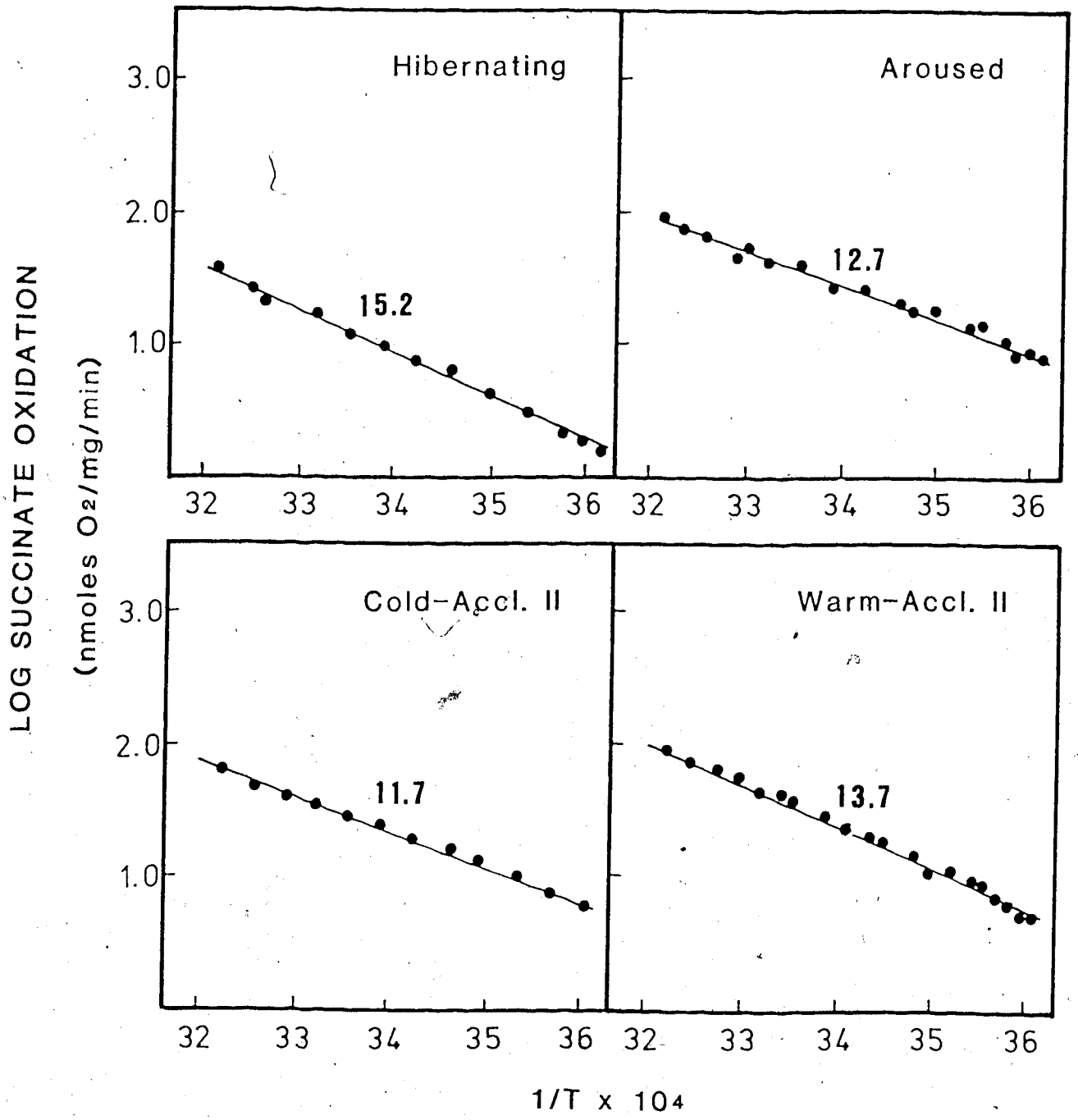


Figure 2.3. Arrhenius plots of O_2 consumption as a measure of succinate dehydrogenase activity in mitochondria from Richardson's ground squirrels which do not show an apparent transition in enzyme function. Each point is the mean of several individuals (see Table 2.1 for (n)). The number beside each line is the Arrhenius activation energy.

Table 2.1. Arrhenius characteristics of respiration-coupled membrane functions during different phases of the hibernation cycle

Group	Assay	T_c(°C)	E_a (kcal/mole)	(n)
Spring (field)	O_2	12	8.5±1.0	(5)
	Ca^{2+}	11	9.6±1.8	(5)
	H^+	13	10.0±2.3	(5)
Summer (field)	O_2	21	9.2±2.0	(6)
	Ca^{2+}	20	9.1±1.0	(6)
	H^+	20	9.0±1.6	(6)
Warm (20° C)-Acclimated (Group I)	O_2	13	9.6±2.1	(7)
	Ca^{2+}	13	8.9±2.0	(7)
	H^+	13	9.1±1.0	(7)
Warm (20° C)-Acclimated (Group II)	O_2	-	13.7±1.4	(5)
	Ca^{2+}	-	13.7±2.2	(5)
	H^+	-	14.2±2.8	(5)
Cold (4° C)-Acclimated (Group I)	O_2	11	8.6±0.9	(4)
	Ca^{2+}	10	7.9±1.9	(4)
	H^+	10	8.4±0.9	(4)
Cold (4° C)-Acclimated (Group II)	O_2	-	11.7±0.5	(4)
	Ca^{2+}	-	9.8±0.9	(4)
	H^+	-	12.6±1.7	(4)
Hibernating	O_2		15.2±1.1	(8)
	Ca^{2+}	-	17.3±2.4	(8)
	H^+	-	17.2±1.1	(8)
Aroused	O_2	-	12.7±2.5	(9)
	Ca^{2+}	-	14.4±3.9	(9)
	H^+	-	13.0±3.1	(9)

Values for activation energy (E_a) are given as mean kcal/mole ± S.D., while those for the critical temperature (T_c) are given as the mean.
(n) = number of individuals used.

they were also significantly lower than the Ea's determined in hibernating animals ($P<0.005$)(Table 2.1). The final group, animals trapped in the field in spring after emergence from hibernation, showed nearly identical Arrhenius profiles to cold-acclimated animals with non-linear plots (CA(l)); i.e., plots became non-linear near a mean *Tc* of 12° C (Fig. 2.2).

The absolute rates of oxygen consumption for succinate-supported respiration at the temperature extremes experienced during hibernation are listed in Table 2.2. Spring and cold-acclimated (CA(l) only) appeared quite similar in all respects and thus the spring group can be considered, for the purposes in this study, as being in a cold-acclimated, non-hibernating phase. Further, the mean daily air temperature at time of capture was only 4-5° C and burrow temperatures would not have been appreciably warmer than the 0-2° C typically observed in winter (Wang, 1979), thus comparable to the experimental conditions of the cold-acclimated animals. The major difference between the spring and cold-acclimated and summer and warm-acclimated groups was the higher respiration rate at 37° C in the latter two groups. However, very little difference was found in respiration rates at 4° C in all four groups. In contrast, the rates of O_2 consumption were greatly depressed at all assay temperatures (4-37° C) during hibernation compared to aroused animals ($P<0.005$), even though neither group displayed a discernible Arrhenius discontinuity. While the rates of O_2 consumption at 37° C in aroused animals was the same as in summer and warm-acclimated groups, at 4° C the rate was higher than any other groups examined (Table 2.2).

Table 2.2. Absolute rates of oxygen consumption of liver mitochondria at the temperature extremes experienced during the hibernation cycle.

Group	37° C (nmoles O_2/mg)	4° C (nmoles O_2/mg)	(n)
Spring (field)	53.7±12.2	5.0±0.9	(5)
Summer (field)	76.4±11.6	5.0±1.0	(6)
Warm (20° C)-Acclimated (I)	86.5±9.3	5.7±1.0	(7)
Warm (20° C)-Acclimated (II)	79.9±10.8	5.0±0.7	(5)
Cold (4° C)-Acclimated (I)	66.9±10.6	6.0±0.7	(4)
Cold (4° C)-Acclimated (II)	56.5±13.6	5.9±1.1	(4)
Hibernating	32.9±7.1	1.7±0.5	(8)
Aroused	79.4±15.4	8.0±2.3	(9)

Rates of O_2 consumption are mean ± S.D.

D. Discussion

Non-linear Arrhenius plots of several enzyme activities from membranes of non-hibernators have been shown to occur as temperature is reduced. Such non-linear plots have been documented for heart microsomes (McMurchie *et al.* 1973) as well as for enzymes of mitochondrial electron transport (McMurchie, *et al.*, 1973; Lee and Gear, 1974; Lenaz *et al.*, 1972). In all cases an apparent increase in activation energy was evident below a calculated "break" temperature. A crucial observation is that similar non-linear Arrhenius plots occur in some mammalian species capable of but not prepared for hibernation. The absence of any breaks and a constant activation energy in animals hibernating at a T*b* of 2-4° C thus suggests either a temperature induced change or a seasonal alteration in the catalytic activity of these enzymes.

In the present study non-linear Arrhenius plots of O_2 consumption in summer-active ground squirrels indicates similar thermal behaviour to that of mitochondria from non-hibernators such as the rat (Kemp, *et al.*, 1969; Lee and Gear, 1974) or the rabbit (McMurchie *et al.*, 1973). Corresponding non-linear plots of H^+ efflux and Ca^{2+} uptake further substantiates the thermal behaviour of succinate dehydrogenase, since the rate at which protons are extruded from the inner mitochondrial membrane is dependent upon the rate at which this enzyme supplies reducing equivalents to the electron transport chain (Wikstrom and Krab, 1980). As a consequence of the tight coupling of ion transport to respiration (Nicholls, 1982), the rate of Ca^{2+} uptake is directly linked to the membrane potential gradient invoked by H^+ extrusion. The lowering of the Tc from 20°C in summer animals to 11-13° C in winter animals (both warm- and cold-acclimated) suggests a preparative change for hibernation. The observation that some warm- and cold-acclimated animals (WA(II) and CA(II), Fig. 2.2; Table 2.1) showed no transition down to 4° C is unique in that these individuals were euthermic and had not experienced a prior bout of torpor. Coincidentally, all of these animals were either in the steep ascending or plateau phase of a body weight gain cycle, characteristic of a transition from the non-hibernating to hibernating phase (Glass and Wang, 1979). Thus it seems apparent that a period of deep torpor (T*b* 2-5° C) is not a prerequisite for the exhibition of linear Arrhenius plots. This further suggests that the preparation for hibernation culminates in the abolition of any thermally induced transition in the temperature dependence of respiration-linked activity

over the range of *Tb* s typically encountered during hibernation. The fact that all hibernating and aroused animals in this study yielded completely linear plots for all three membrane-linked functions is evidence that the lowering of the thermal transition of succinate dehydrogenase activity achieved during preparation for hibernation is maintained throughout the hibernating phase of the animal, despite the wide excursions of Tb between hibernation and euthermia. The thermodynamic considerations of a single activation energy during the hibernating phase are not trivial. By virtue of the exponential relationship of rate to *Ea*, even an apparently small increase in *Ea* will be reflected in a considerable decrease in velocity. We can assume therefore that during hibernation the enthalpies of activation for the enzymic reactions in question are invariant with temperature.

Several studies have attempted to correlate enzyme activity with the phase state of membrane lipids. Arrhenius breaks seen at 20 ° C for Ca^{2+} accumulation and ATPase activity of sarcoplasmic reticulum coincided with an increase in spin-probe motion and a decrease in ordering of acyl chains above the *Tc* (Inezi, *et al.*, 1973). However, using conventional and ST-EPR, Squier *et al.* (1984) have recently demonstrated that no thermotropic transitions occur in the lipid hydrocarbon chains of sarcoplasmic reticulum, even though spin probes may detect "breaks" in enzymatic and protein rotational mobility. For example, breaks detected for mitochondrial succinate oxidation, McMurchie *et al.* found corresponding biphasic plots for heart rate and spin labelled membrane preparations. More recently it has been shown that diet-induced changes in membrane phospholipids can alter the thermal behaviour of succinate: cytochrome *c* reductase in rats (McMurchie *et al.*, 1983). With respect to hibernators no clear relationship has been demonstrated between shifts in the thermal response of enzyme activity and membrane lipid alterations. For example, although there was no correlation with breaks in Arrhenius plots of intramitochondrial protein movement, Cremel *et al* (1979) observed a trend toward increasing levels of polyunsaturated fatty acids in liver mitochondria in the European hamster prior to hibernation. In the 13-lined ground squirrel a similar shift was seen in the unsaturation index of erythrocyte membrane lipids during cold acclimation and a potentiation of these changes in the hibernating state (Rotermund and Veltman, 1981). As well, the alterations of membrane phospholipid classes (Aloia, 1979, 1981) and enhanced spin-label mobility (Raison *et al.*, 1981) seen in

torpid squirrels suggest a membrane lipid-mediated adjustment for hibernation. Lipid involvement in the form of a phase separation has been proposed to explain nearly identical downward shifts in Tc seen in five diverse enzymes in liver plasma membranes from hibernating hamsters (Houslay and Palmer, 1978). It appears unlikely that independent but equivalent changes in reaction mechanisms, rate limiting steps, or protein conformation could influence Arrhenius plots in this manner.

A body of evidence also exists which suggests that enzyme functions are unrelated to membrane lipid structure. Lenaz *et al* (1972) showed that breaks in Arrhenius plots of membranous enzymes in intact mitochondria fall at different temperatures and suggest caution in their correlation with phase transitions of membrane phospholipids. Perhaps the strongest evidence against a causal relation between enzyme activity and membrane phase transitions is the observation that while breaks are seen near 21° C in mitochondrial succinate dehydrogenase activity, calorimetric analysis shows that the bulk lipid liquid crystalline-gel phase transition occurs near 0° C in rat liver mitochondria. (Blazyk and Steim, 1972). Conversely, when the calorimetric data is coupled with freeze-etch examination of the inner mitochondrial membrane, it is clear that oxidative phosphorylation is critically dependent upon lipid-protein interaction (Hackenbrock, 1977).

The significant depression in mitochondrial O_2 consumption during hibernation follows a trend seen in other hibernating species. Depressed respiration of liver mitochondria has been observed in hamsters (Liu *et al.*, 1969; Roberts *et al.*, 1973) and in ground squirrels (Daudova, 1968; Liu *et al.*, 1969), although in one instance (Daudova, 1968) washing the mitochondria removed this inhibition. When mitochondria from hibernating squirrels were uncoupled with salicylamide (which inhibits phosphorylation but not respiration), the maximal respiratory rate became higher than that in active animals (Shug *et al.*, 1971). This led to the interpretation that the depressed respiration seen in hibernation could be the result of an inhibition of nucleotide permeation (Willis, 1982). In the present study any rate limiting step in substrate utilization prior to succinate is excluded by the choice of succinate as substrate and by the use of rotenone, which blocks any endogenous NAD-linked substrates. Further, respiration proceeded in the absence of exogenous or endogenous ADP (ATPase was inhibited by oligomycin). Thus, the depression would appear to occur at the level of succinate dehydrogenase. It is

significant that the depressed O_2 consumption is released upon arousal, this reversal apparently not being related to a thermotropic transition since only linear Arrhenius plots are evident in the hibernating and aroused states. As respiration was lower at all assay temperatures (4-37° C), it would appear that the phenomenon is not a consequence of low temperature but may instead reflect some other influence on the activation state of succinate dehydrogenase.

The overall implication of this investigation is that the Richardson's ground squirrel progresses through several physiological-biochemical states during a typical annual hibernation cycle. Each phase is characterized by the thermal behaviour of membrane-associated bioenergetic activity, each membrane state appearing to be attained independent of acclimation or body temperature.

III. STOICHIOMETRY OF H^+ EFFLUX TO RESPIRATION-DEPENDENT Ca^{2+} UPTAKE AND OXYGEN CONSUMPTION IN LIVER MITOCHONDRIA

A. Introduction

With the general acceptance of the chemiosmotic theory it is recognized that the conservation of redox energy in mitochondria is a consequence of the net translocation of protons across the inner, energy transducing membrane (Mitchell, 1978). Fundamental to this concept is the need for a topologically closed, insulating membrane and a stoichiometric coupling of proton efflux to cation uptake and oxygen consumption. Specifically, the translocation of H^+ across the inner mitochondrial membrane generates both an electrical and a chemical gradient which may subsequently drive the uptake of Ca^{2+} (Rottenberg and Scarpa, 1974). It has been firmly established that Ca^{2+} entry is via an electrophoretic uniport process in which one Ca^{2+} ion is taken up to compensate for each pair of H ions ejected during electron transport (Reynafarje and Lehninger, 1977). Similarly, the electroneutral entry of Ca^{2+} results in a stoichiometric consumption of oxygen with an observed Ca^{2+} / O ratio near 4 (Brand *et al.*, 1976). A second, less well-understood carrier exists which catalyzes a continuous efflux of Ca^{2+} from the mitochondrial matrix. It is apparent that the uptake and efflux pathways operate in synchrony to allow a cycling of Ca^{2+} between the cytosol and matrix (Nicholls and Crompton, 1980), with the rate of efflux determining the regulation of Ca^{2+} distribution by the mitochondria.

The physiological implications of respiration-linked calcium regulation are evident for the maintenance of cellular homeostasis, but especially so when perturbations to the system occur. One such perturbation which has not been well studied is the influence of temperature on the bioenergetics of mitochondrial respiration and its role in Ca^{2+} regulation. The temperature dependence of Ca^{2+} transport may be of particular significance in those mammals which exhibit seasonal hibernation, a phenomenon characterized by depressed metabolism and body temperature. During hibernation body temperature is maintained near 0° C for several weeks, interspersed by periods of arousal during which body temperature is restored to normal (37° C) within a few hours (Wang, 1978). Such wide temperature excursions without any apparent disruption in metabolic

integrity suggest that cellular functions dependent upon strict Ca^{2+} regulation are retained. In contrast, mitochondrial respiration is thought to be subject to thermally induced impairment in non-hibernating species (Lee and Gear, 1974) and in hibernating species in their active season (i.e. summer) (Raison and Lyons, 1971). An apparent abrupt increase in the activation energy (*Ea*) for succinoxidase occurred below a critical temperature (*Tc*) near 21° C in active ground squirrels but was constant between 4 and 37° C during hibernation and thus invariant with temperature (Raison and Lyons, 1971; Pehowich and Wang, 1981). Several attempts have been made to correlate enzymatic differences between active and hibernating states with changes in either lipid composition (Cremel *et al.*, 1979; Aloia, 1980) or in the thermotropic ordering of membrane lipids (Keith *et al.*, 1975; Raison *et al.*, 1981). While it does appear that the phospholipid and fatty acid components of membranes are different between the two states, to date no direct relationship between membrane function and lipid properties can be ascribed to the phenomenon of hibernation.

In view of the differences seen in the rate-temperature relationship between active and hibernating squirrels, we have examined the stoichiometric coupling of Ca^{2+} uptake to proton ejection and oxygen consumption in the two physiological states. Our approach of evaluating the H^+ / Ca^{2+} and Ca^{2+} / O ratios of mitochondrial electron transport is to determine if; 1) the mechanism of respiration-linked Ca^{2+} transport is maintained at all body temperatures experienced during hibernation, and 2) the changes in thermal sensitivity seen in mitochondrial respiration in active animals are reflected in an uncoupling of Ca^{2+} uptake and electron transport.

The results described herein suggest that the capacity for the precise regulation of cellular Ca^{2+} is retained after several weeks of hibernation at body temperatures as low as 4° C, as H^+ / Ca^{2+} and Ca^{2+} / O ratios were constant over the entire body temperature range seen in a typical hibernation bout. There does not appear to be a thermally induced disruption of the inner mitochondrial membrane in mitochondria from summer-active animals, as constant H^+ / Ca^{2+} and Ca^{2+} / O ratios were observed at all temperatures despite a dramatic change in rate functions (i.e., a non-linear Arrhenius plot) in the temperature region below 21° C.

B. Materials and Methods

Animals

Richardson s ground squirrels (*Spermophilus richardsonii*) were trapped near Edmonton in June and July. Some of the animals were assayed within one week of capture and others maintained in a controlled environment chamber at 4° C under constant darkness to initiate hibernation. Hibernating animals were killed in November-December, after at least three days into a well-established hibernation bout.

Isolation of mitochondria

Liver mitochondria were isolated from active (T_b=37° C) and hibernating (T_b=4° C) squirrels in an ice-cold medium consisting of 0.25 M sucrose, 0.5 mM EGTA, 0.5% BSA, and 3.0 mM HEPES, pH 7.4 (Pehowich and Wang, 1984; Chapter 2). The mitochondria were washed twice in 0.25 M sucrose, 2.0 mM HEPES, pH 7.4, and resuspended to a final concentration of 60-100 mg protein per ml. The protein content of the mitochondrial suspension was determined after solubilization of an aliquot in sodium dodecyl sulphate (Markwell *et al.*, 1978).

Measurements of H^+ ejection, Ca^{2+} uptake and oxygen consumption

Reactions were carried out in a thermostatted glass chamber in a total volume of 5 ml of medium consisting of 120 mM sucrose, 60 mM KCl, 2 mM HEPES, pH 7.4, and 2 nmoles of rotenone per mg protein. Any spurious reuptake of H^+ via a phosphate symport was prevented by the addition of 40 nmoles per mg protein of N-ethylmaleimide (NEM). Any proton ejection which may have resulted from the hydrolysis of endogenous ATP was eliminated by the inclusion of 1 µg of oligomycin per mg protein. After an initial 2 min incubation, $CaCl_2$ was added to the medium to a final concentration of 60 nmoles per mg of mitochondrial protein and electron flow was intiated by the addition of succinate as electron donor at a final concentration of 2 mM. Rates of H^+ ejection, Ca^{2+} uptake and oxygen consumption were measured simultaneously as described previously (Pehowich and Wang, 1984; Chapter 2) with appropriately calibrated electrodes. The slopes of the initial, linear portions of H^+, Ca^{2+} and O_2 traces were used to calculate the H/Ca^{2+} and

Ca^{2+} / O ratios. Rates were determined at 2 °C intervals between 4 and 37° C.

C. Results

Measurement of rates of H^+ ejection, Ca^{2+} uptake and oxygen consumption

The initial period of aerobic incubation in the presence of rotenone was sufficient to inhibit any endogenous respiration and to completely de-energize the mitochondria. At 25° C the initiation of electron flow with a pulse of succinate resulted in a rapid ejection of protons into the reaction medium with a corresponding decrease in the Ca^{2+} content of the medium as the ion was accumulated . The rate of H^+ ejection and Ca^{2+} uptake became constant and linear within 2-3 sec. Oxygen consumption took longer to become linear (4-5 sec), however, all three rates were simultaneously linear for several seconds thereafter. After 12-15 sec the rate of H^+ ejection declined rapidly, as did the rate of Ca^{2+} uptake, however, oxygen consumption continued for several seconds longer and declined more slowly. The slower decline in oxygen consumption reflected a back leakage of H^+ into the mitochondrial matrix which stimulates respiration. At higher temperatures rates became linear almost immediately and the duration of the linear phase was shorter. At lower temperatures the time taken to reach linearity was correspondingly longer to the extent that at 4° C it took several minutes to accumulate added Ca^{2+}.

Arrhenius plots of H^+ ejection, Ca^{2+} uptake and oxygen consumption

The temperature dependence of H^+ ejection, Ca^{2+} uptake and oxygen consumption in active and hibernating animals are revealed in Arrhenius plots of rate *vs* temperature (Fig. 3.1; 3.2). Plots of activities in mitochondria from active animals (T_b=37 °C) were initially linear but became non-linear below 21-23°C. An average E_a of 9.3 kcal / mole was calculated for the linear temperature region of the H^+ ejection plot and the E_a's for Ca^{2+} uptake and oxygen consumption were not significantly different. Animals killed in deep torpor (T_b=5° C) showed no apparent change in the temperature dependence of H^+ ejection, Ca^{2+} uptake or oxygen consumption, Arrhenius plots for all three functions were linear with only a single E_a between 4 and 37° C (Fig. 3.2). The E_a's in hibernating animals were not significantly different from each other but were significantly different ($P<.005$) from the E_a's for H^+ ejection, Ca^{2+} uptake and oxygen consumption in the upper linear temperature region in active animals.

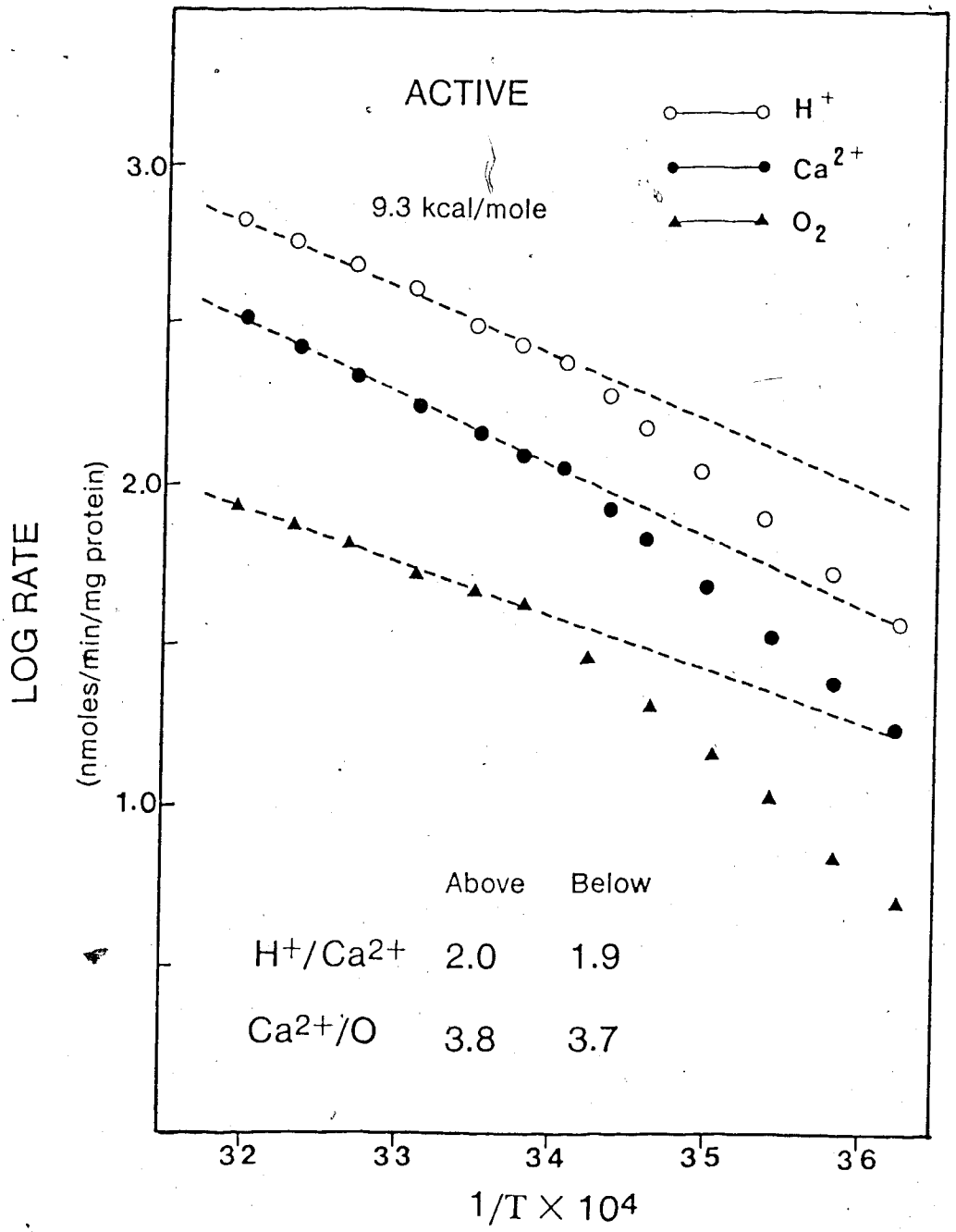


Figure 3.1. Arrhenius plots of H^+ efflux, Ca^{2+} uptake and oxygen consumption in liver mitochondria from active Richardson's ground squirrels.

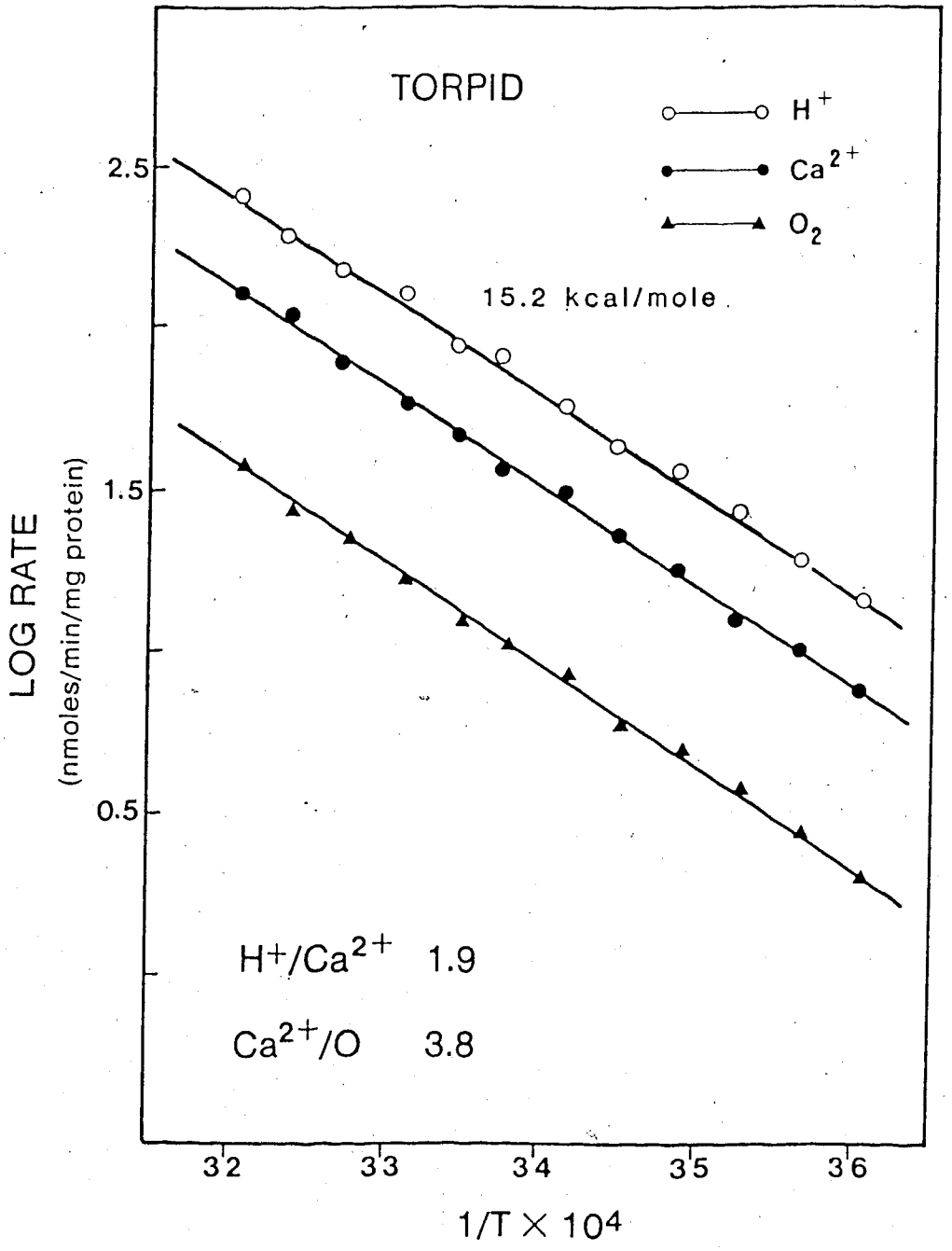


Figure 3.2. Arrhenius plots of H^+ efflux, Ca^{2+} uptake and oxygen consumption in liver mitochondria from hibernating Richardson's ground squirrels.

Stoichiometry of respiration: H^+ / Ca^{2+} and Ca^{2+} / O ratios

The rate measurements during the linear phase of H^+ ejection, Ca^{2+} uptake and oxygen consumption represented the initials rates of these activities in previously energy-depleted mitochondria. Because the mitochondria were de-energized there was no pre-existing membrane gradients to bias the calculation of the H^+ / Ca^{2+} (nmoles H^+ ejected per nmole Ca^{2+} accumulated) or Ca^{2+} / O (nmoles Ca^{2+} accumulated per nmole O consumed) quotients once respiration was initiated. At 37° C the H^+ / Ca^{2+} quotient was 1.9 for active squirrels based on 1170 nmoles H^+ ejected per mg/min and 598 nmoles Ca^{2+} taken up per mg/min. This ratio varied between 1.8 and 2.1 between 37 and 22° C but was not different from the average quotient of 2.0 over this temperature range. Between 20 and 4° C (below Tc) the mean ratio was 1.9; at 4° C the H^+ ejection rate was 83.8 nmoles per mg/min, the Ca^{2+} uptake rate 44.6 nmoles per mg/min (Table 3.1). A nearly constant Ca^{2+} / O rate quotient was also apparent at all temperatures for active animals. Oxygen consumption was 79.4 nmoles O_2 per mg/min at 37° C and 5.7 nmoles per mg/min at 4° C yielding Ca^{2+} / O ratios of 3.8 and 3.7, respectively (Table 3.1). The rate quotients at all other temperatures were not significantly different from these values.

The absolute rates of H^+ ejection, Ca^{2+} uptake and oxygen consumption were considerably lower at all temperatures in hibernating animals (Table 3.1), however, the stoichiometry of these processes was unchanged. At 37° C the rate of H^+ ejection was 412.5 nmoles per mg/min compared to 221.8 nmoles per mg/min for Ca^{2+} uptake resulting in a H^+ / Ca^{2+} ratio of 1.9. At 4° C the ratio was 1.8. Similarly, little variation in the Ca^{2+} / O ratio was observed; the mean value was 3.8 between 37 and 4° C.

Table 3.1. Absolute rates of mitochondrial H^+ ejection, Ca^{2+} uptake and oxygen consumption at 37° and 4° C in active and hibernating Richardson's ground squirrels.

Hibernation State		37° C		4° C
Active (n=13)	[1]H^+	1,170.4±6.28	H^+	83.8±0.98
	[2]Ca^{2+}	598.0±4.24	Ca^{2+}	44.6±1.48
	[3]O_2	79.4±2.02	O_2	5.7±0.29
Hibernating (n=13)	H^+	412.5±3.86*	H^+	24.5±1.10*
	Ca^{2+}	221.8±1.90*	Ca^{2+}	12.8±0.99*
	O_2	29.5±2.18*	O_2	1.7±0.22*

[1]Rates are nmoles per mg protein/min., means ± SE.

[2]Rates are nmoles per mg protein/min., means ± SE.

[3]Rates are nmoles per mg protein/min., means ± SE.

*Statistically significant difference between active and hibernating groups ($P<0.001$). Determined by two-tailed Student's t-test.

D. Discussion

The coupling of transport to an energy-yielding reaction is basic to the regulation of Ca^{2+} by mitochondria. Only H^+ ions appear to be actively translocated in energized mitochondria and the transport of all other ions are chemically or electrically coupled to this process (Mitchell, 1966).

This study demonstrated that there are differences in the activity of the electron transport chain between active and hibernating ground squirrels, these differences being reflected in the Arrhenius activation energies and absolute rates of H^+ ejection, Ca^{2+} uptake and oxygen consumption. Similar differences have previously been demonstrated for succinate oxidase activity in active and hibernating ground squirrels (Liu et al., 1969; Raison and Lyons, 1971; Roberts *et al.*, 1972; Chapter 1; Chapter 2). We assume that the activities measured in this study relate directly to the respiratory chain and our assumptions are likely valid in that the Km for succinate for both proton efflux and oxygen consumption has been shown to be identical,i.e., 0.20 mM (Reynafarje *et al.*, 1976). This means that both functions are a consequence of succinate oxidase activity, a fact that was reinforced by our observation that no net ion movement or oxygen consumption occurred until succinate was added.

Under conditions which prevent the inward movement of phosphate and endogenous substrate utilization, a H^+ / Ca^{2+} ratio near 2 is expected when electrons traverse the two energy-conserving sites available with succinate as substrate (Brand et al., 1976). An electroneutral exchange for Ca^{2+} is the consequence of a proton electrochemical gradient developed during respiration, the rate of Ca^{2+} uptake being correlated with the magnitude of the membrane potential (Akerman, 1978). This is precisely what we have observed at all temperatures in both active and hibernating animals. A tight coupling of Ca^{2+} uptake is maintained over the widest possible physiological temperature range and during two different physiological states. The observed maintenance of a stoichiometric relationship of Ca^{2+} uptake with the proton-generated membrane potential is of particular importance in that it indicates that the inner mitochondrial membrane is intact after several days of low temperature during hibernation. It is also of note that this integrity is not disrupted by the isolation procedure and storage for several hours at 0° C.

A H^+ / Ca^{2+} ratio near 2 at all temperatures in active animals indicates that a dramatic departure from euthermic conditions does not compromise the transport properties of the inner mitochondrial membrane. A strict rate quotient is maintained despite an apparent change in the temperature dependence of succinate-driven respiration, an observation which suggests that non-linearities in Arrhenius plots may not necessarily be a result of membrane damage. This is particularly evident when we realize that the thermodynamic consequences of membrane cooling are completely reversible, i.e., the mitochondria are exposed to 0 to 4° C for several hours during preparation and storage, yet are viable upon rewarming to 37° C. We assume that the E*a* calculated for the upper linear temperature region of the Arrhenius plot represents the functional E*a* for the active physiological state because these animals are normally euthermic (T*b*=37°C) at this time.

While similar arguments can be made for the maintenance of a Ca^{2+} / O ratio near 4 in active and hibernating animals, these observations also uniquely demonstrate that oxygen consumption is not uncoupled by Ca^{2+} uptake, a situation which can occur in the presence of phosphate (Leblanc and Clauser, 1974), but is prevented by adequate levels of mitochondrial Mg^{2+} and ADP (Cheah and Cheah, 1978). Also, Ca^{2+} can promote other deleterious effects such as swelling (Hunter et al., 1976) and phospholipase A_2 activation (Scherphof et al., 1972), both of which increase membrane permeability. Apparently permeability changes do not develop as a consequence of the wide thermal excursions seen in a hibernation bout as they would be reflected in H^+ / Ca^{2+} and Ca^{2+} / O ratios. Swelling and concomitant permeability changes increase proton leakage and thus lower the H^+ / Ca^{2+} ratio (leakage of H^+ would disipate the membrane potential and thus reduce the inward movement of Ca^{2+}).

A marked depression in respiration-linked activity during hibernation has been well documented (Willis, 1980; also see Chapter 2). While no tangible explanation for this inhibition is available, the ion ratios observed in this study suggest that the depression of electron transport activity may be an adaptation related to energy conservation and not a thermal impairment of the energy transducing membrane. The energy needs during hibernation are certainly reduced and it would appear that energy conservation derived from electron transport can be curtailed and yet associated mechanisms such as Ca^{2+} uptake kept intact.

Another aspect of mitochondrial respiration during hibernation which has not been clearly resolved is the observed shift in the apparent energy of activation for the succinate oxidase pathway. While there is an apparent alteration in the kinetic or thermodynamic aspects of respiration at low temperature in animals not capable of or not prepared for hibernation, there is no adequate explanation as to why the *Ea* is significantly higher in the hibernating state than that seen at euthermia in the non-hibernating state. Generally, a transition in the temperature dependence of *Ea* corresponds to the midpoint of a thermotropic change in the state of a system (Silvius and McElhaney, 1981), but, whether an intrinsic conformational change in the rate-limiting enzyme(s) or a conformation change induced by an alteration in its lipid environment is influencing reaction rates is still a point of discussion. The implication is that whatever factors influence the overall activity of the energy transducing membrane in hibernators do not necessarily determine the intrinsic stoichiometry of Ca^{2+} uptake.

A critical role of mitochondria in general is the maintenance of Ca^{2+} homeostasis, attained through the precise regulation of energy dependent uptake and cycling across the inner membrane (Nicholls and Crompton, 1980). The present study has demonstrated that liver mitochondria in a mammalian hibernator retain the capacity to exert a strict control over intracellular and intramitochondrial Ca^{2+} during long periods of torpor, characterized by a pronounced depression of body temperature and metabolism. Such control is vital not only for survival during hibernation but also for the energetically demanding rewarming to euthermia which is an integral part of mammalian hibernation.

IV. THERMOTROPIC BEHAVIOR OF INNER MITOCHONDRIAL MEMBRANE IN DIFFERENT PHASES OF HIBERNATION CYCLE

A. Introduction

In the first three chapters of this study we have documented the occurrence of thermally induced transitions in the rate functions of some membrane-bound activities, i.e., oxidative phosphorylation and Ca^{2+} uptake in liver mitochondria from an active hibernator. When animals were killed in hibernation no such transition was discernible as inferred from linear Arrhenius plots. Our knowledge of Arrhenius behaviour tells us that at best, plots of the activities of membrane-bound enzymes can only predict the mid-point of a thermotropic change in the state of a system and that sharp Arrhenius breaks can arise in systems which do not undergo phase transitions (McElhaney, 1982).

The impetus in recent investigations of the molecular aspects of hibernation has been toward correlating increased cold tolerance, inferred by linear Arrhenius plots, with a modification of membrane lipid composition (e.g., Keith *et al.*, 1974; Charnock *et al.*, 1980; Raison *et al.*, 1981). A lowering of the liquid crystalline-gel phase transition temperature has been proposed as a vital part of the overall preparation for hibernation. Obviously there is a need for the critical analysis of the thermotropic properties of membranes in hibernators in order to determine if increased cold tolerance is associated with the bulk lipid phase behaviour or if we have to shift our attention toward other membrane lipid events such as phase separations or boundary lipid domains (Aloia, 1979).

Differential scanning calorimetry is regarded as the most powerful technique for the analysis of lipid phase transitions in biological membranes (McElhaney, 1982). Unlike techniques such as electron spin resonance, which detect only the midpoint of a thermotropic phase transition, high sensitivity DSC can document the entire course of a phase transition and reports the "average" thermotropic behaviour. As well, other physical methods for studying membrane structure and dynamics require perturbation of the membrane by a probe and suffer from errors in interpretation (Schreier *et al.*, 1972). In the only study employing DSC in the examination of membranes from hibernator tissue, Charnock *et al.* (1980) found that a phase transition occurred near 26°C in myocardial membranes from summer-active ground squirrels and the transition was lowered to 16°C

in hibernating animals.

While nuclear magnetic resonance spectroscopy in general suffers from low sensitivity of detection, it has the advantage of being able to study the membrane directly. The ^{19}F nucleus appears to be a probe particularly suited to examining orientational order and motional rates in biological membrane fatty acyl-chains by virtue of its high sensitivity and low natural abundance (McDonough *et al.*, 1983). The ^{19}FNMR line shape can be interpreted in terms of the orientational order parameter, *Smol*, of the hydrocarbon long molecular axis. *Smol* represents the time-averaged angular excursion of a methylene segment from the bilayer normal and thus is an index of the lipid mobility within the bilayer. To date NMR has not been used to characterize membranes in hibernators.

Fluorescence spectroscopy, which is based on the anisotropy of the polarization ratio of a fluoroprobe, is highly sensitive although it too suffers from the disadvantage of requiring extrinsic probes. Fluorescence polarization of 1,6-diphenyl-1,3,5 -hexatriene (DPH) has been used sucessfully to observe quantitative differences in the fluidity of synaptic membranes and mitochondria from thermally acclimated goldfish (Cossins and Prosser, 1982). When the same technique was used to examine brain synaptosomes in hamsters, no differences were found between active and hibernating animals and neither was different from the rat (Cossins and Wilkinson, 1982).

This study is an examination of the thermotropic behaviour of the inner mitochondrial membrane from ground squirrels in different hibernating states using the three described techniques; DSC, ^{19}FNMR spectroscopy and DPH fluorescence polarization spectroscopy. The inner mitochondrial membrane was chosen for examination as it is the energy-transducing membrane of the mitochondrion and the loci of the activities exhibiting seasonal changes in temperature dependence (Chapters 1 and 2).

B. Materials and Methods

Animals

Richardson's ground squirrels were collected and kept as described in Chapter 2. Differential scanning calorimetry was performed on the liver inner mitochondrial membranes of adult animals trapped in early August, cold-acclimated animals killed in October, hibernating animals, animals aroused from hibernation, and animals trapped in April, about two weeks after emergence from hibernation.

Isolation of inner mitochondrial membrane

Whole mitochondria were isolated as described in Chapter 2 and the inner membrane-matrix fraction (mitoplasts) was prepared according to the procedure of Schnaitman and Greenawalt (1968). The final whole mitochondrial pellet was resuspended in 70 mM mannitol, 120 mM sucrose, 2 mM HEPES, pH 7.4, and 0.5 mg BSA per ml to a final concentration of 100 mg protein per ml. Digitonin (Sigma) was dissolved in one volume of medium by heating the medium to 100° C, and then cooled on ice. The digitonin solution was added to the mitochondria stock to a final concentration of 0.15 mg per mg. of protein. The mitochondrial suspension was stirred at 0° C for 20 min and then homogenized with a Potter-Elvehjem tissue grinder with a tight-fitting glass pestle. The inner mitochondrial membrane fraction was separated by centrifugation at 12,000 g for 15 min. Any dark material on the surface of the pellet was washed off with fresh medium and the pellet resuspended and centrifuged again. The final pellet was resuspended in 50 mM Tris-HCl, pH 7.4, and centrifuged at 20,000 g for 10 min. The purified inner membrane fraction was then frozen quickly in dry ice-acetone and lyophilized at -70° C. Half of each preparation was used for calorimetry and lipids were extracted from the remaining portion. Membranes to be used for calorimetry were flushed with dry N_2 and sealed in teflon-lined screw cap tubes and stored at -28° C until analysis.

The purity of the inner membrane preparations was assessed by measuring the specific activity of monoamine oxidase, an outer membrane marker. A portion of the preparation was solubilized in 0.1% polyoxyethylene ether (Sigma) and the specific activity of monoamine oxidase determined as nmoles of benzylamine oxidized per mg of protein.

Oxidation was measured as the change in absorbance at 240 nm in 0.05 M phosphate buffer, pH 7.4, containing 2% benzylamine.

Differential scanning calorimetry

Lyophilized inner membrane preparations were rehydrated in 100-150 *ul* of 50% ethylene glycol in 0.05 M Tris-HCl, pH 7.4, which had been flushed with N_2. The membranes were rehydrated at room temperature to ensure that hydration occurred when the membrane lipids were in the liquid- crystalline state. When the membranes were fully dissolved they were sealed in tubes, flushed with N_2 and kept on dry ice until scanned. Calorimetric analysis was performed on a Perkin Elmer DSC-2C differential scanning calorimeter equipped with a data acquisition station. Sample pans containing 40-50 mg of membrane and a reference pan containing 50% ethylene glycol were run through at least two heating / cooling cycles (230-340° K) in order to fully hydrate the lipid component and to denature protein. Thermal scans, either heating or cooling, were made at a scan rate of 10° C per min.

^{19}F Nuclear magnetic resonance spectroscopy

Monofluorinated palmitic acid was synthesized as described by McDonough *et al.*(1983) in the form of $CH_3(CH_2)m$-$CHF(CH_2)n$-COOH, where $m + n = 13$. Lyophilized inner mitochondrial membrane preparations were suspended in a buffer consisting of 0.154 M NaCl, 0.05 M Tris-HCl, 20 mM *B*-mercaptoethanol, pH 7.4, and diluted 20-fold with deuterium oxide. Preparations were incubated at 37° C for 30 min under N_2 to incorporate the monofluoropalmitate into the membrane fatty acyl chains. ^{19}F NMR spectra were obtained at 270, 280, 290, 300 and 310° K at 254.025 MHz on a Brucker HXS270 NMR spectrometer essentially as described by Macdonald *et al.* (1983).

DPH Fluorescence polarization spectroscopy

Inner mitochondrial membranes were isolated from summer-active ground squirrels trapped in June and winter-hibernating ground squirrels as described above. Preparations were suspended in 0.25 M sucrose, 2.0 mM HEPES, pH 7.4, and frozen on dry ice under vacuum. The frozen samples were sealed in plastic screw-capped vials under

N_2 and kept at -70° until analysis. Just before spectroscopy, samples were thawed under N_2 and an aliquot suspended in 2.4 ml of 0.05 M phosphate buffer, pH 7.6, to give a $O.D._{500} = 0.1 \pm 0.01$. 1,6-diphenyl-1,3,5-hexatriene was dissolved in tetrahydrofuran under N_2 and a 2 *u*l aliquot added to the membrane suspension to give a final probe: phospholipid ratio of 1:200. The preparations were incubated at 37° C for 15 min to incorporate the probe. Fluorescence polarization was measured using a T-format, photon-counting fluorimeter as described by Cossins and Wilkinson (1982). Samples were scanned from 2 to 40° C at a rate of 1°C per min with polarization values being recorded at each 0.2°C increase in temperature.

C. Results

Differential scanning calorimetry

The presence of 50% ethylene glycol in inner mitochondrial membrane preparations allowed cooling of the samples to 230° K (-43°C). A 25% ethylene glycol medium showed a large exotherm near 235° K (-38°C), indicating a water transition had occurred and so 50% ethylene glycol was routinely used. There did not appear to be any other significant difference in the thermograms of the same preparation using either 25 or 50% ethylene glycol and it was not possible to determine if ethylene glycol depressed the transition temperature, as scans of membranes rehydrated in 0.05 M Tris-HCl only showed large water transitions near 270° K (-3°C) which completely masked the lipid transition.

The inner mitochondrial membranes of summer-active animals exhibited an endothermic transition on heating centered near 271°K (-2°C) and occurring over the temperature range 259°K to 283°K (-14 to +10°C)(Table 4.1). A similar transition was observed in membranes from hibernating animals except that the phase transition midpoint (268°K (-5°C)) and boundaries (255-279 °K (-18 to +6°C)) were shifted downward by 4-5°. In both membranes the width and cooperativity of the lipid gel to liquid-crystalline phase transition were similar. The lipid phase transitions observed were reversible except that a pronounced cooling hysteresis was usually observed, resulting in an apparent downward shift in the position of the phase transition by 12 to 14°K (Fig. 4.2). However, as kinetic limitations and instrumental limitations are more severe in the cooling mode, the heating endotherms are considered to be more reliable estimates of the true phase transition temperatures. Representative heating and cooling thermograms of individual animals are shown in Figure 4.1 and Figure 4.2. Heating and cooling thermograms of aroused, cold-acclimated, warm-acclimated and spring animals were not different from hibernating animals but were all different from summer animals. The analysis of these transitions are summarized in Table 4.1.

Table 4.1 Thermal properties of liver inner mitochondrial membrane from ground squirrels in different phases of hibernation cycle.

	Heating Scan*				Cooling Scan			
	(° K)				(° K)			
Phase	Onset Temp.	Transition Temp.	Final Temp.	dT	Onset Temp.	Transition Temp	Final Temp.	dT
Summer(7)	261.5±0.77	270.4±0.55	280.3±1.45	22.6±1.40	274.9±2.40	266.3±1.38	258.0±1.08	23.9±0.93
Hibernating(7)	260.2±0.96	267.5±0.41[1]	276.5±0.60	21.9±0.95	260.8±0.46[1]	256.0±0.39[1]	242.0±0.86	21.4±1.10
Aroused(4)	261.5±1.22	269.1±0.42	276.8±0.85	19.9±1.20	263.8±1.17[1]	257.9±2.24[1]	247.6±4.61	18.4±1.52
Cold(4)	260.9±1.06	267.6±0.62[2]	276.6±0.73	20.4±1.06	263.9±0.98[1]	256.7±1.12[1]	241.9±0.92	20.2±0.89
Warm(4)	260.5±0.99	268.6±0.72[2]	277.2±0.94	21.0±0.96	262.8±0.88[1]	256.2±1.11[1]	243.3±1.27	20.0±1.18
Spring(4)	260.6±0.63	268.9±0.16[2]	275.6±0.85	18.3±1.62	264.2±0.25[1]	256.3±0.19[1]	243.0±0.79	23.8±0.80

Values are means ± SE.

*Samples were heated or cooled at a scan rate of 10° K/min.

Statistically significant difference from summer group [1](P<0.001); [2](P<0.05). Determined by two-tailed Student's t-test.

dT = Temperature range from onset to end of phase transition.

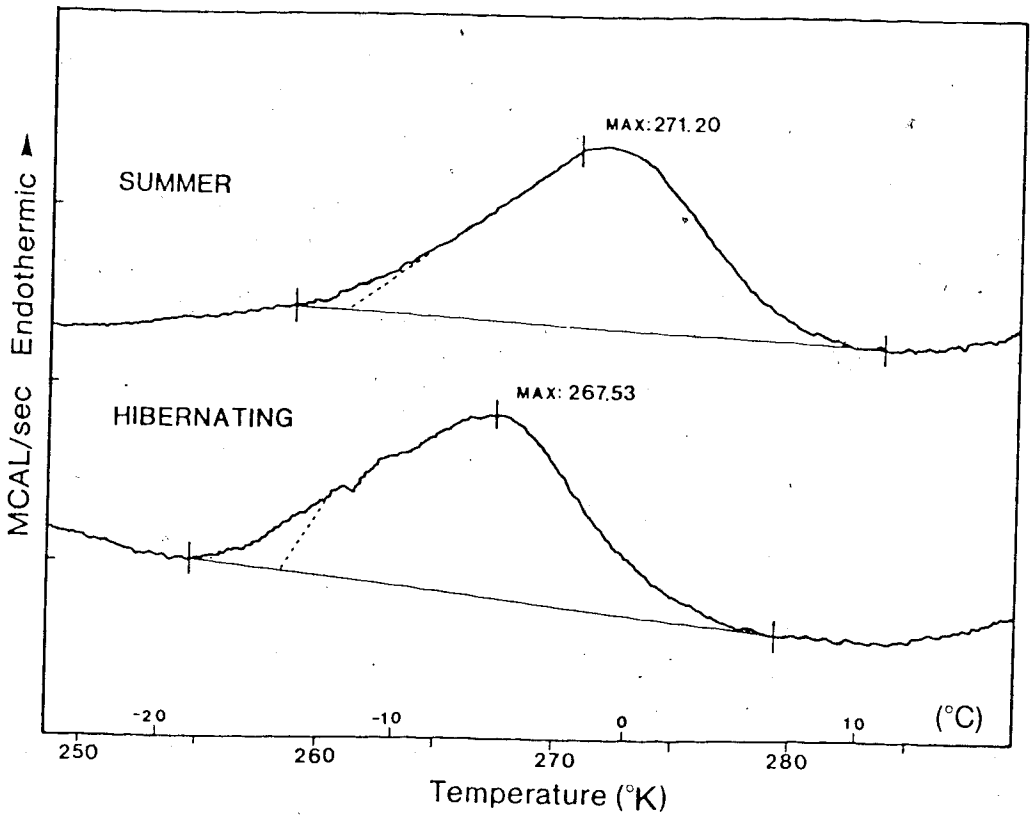


Figure 4.1. Differential scanning calorimetry thermograms of endothermic (heating) phase transitions in liver inner mitochondrial membranes from a summer-active and hibernating Richardson's ground squirrel.

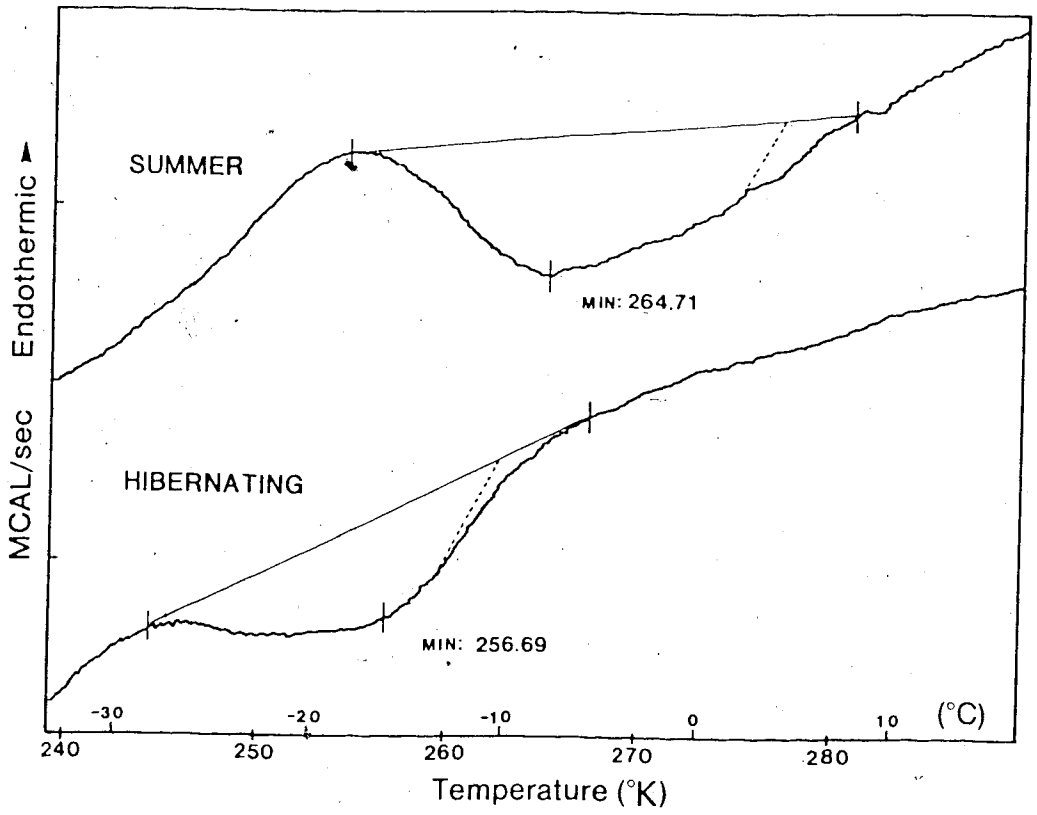


Figure 4.2. Differential scanning calorimetry thermograms from exothermic (cooling) phase transitions of liver inner mitochondrial membranes from a summer-active and hibernating Richardson's ground squirrel.

^{19}F Nuclear magnetic resonance spectroscopy

A general trend seen in the line spectra from summer and hibernating membranes was an increase in line width as temperature decreased from 310 to 270° K (Fig. 4.3), indicating an increase in the order parameter, *Smol*. When order parameter values were plotted against temperature (Fig. 4.4), there was a difference between summer and hibernating animals at 310° K (37° C) indicating a difference in the overall membrane order. Generally, *Smol* approaches unity as lipids become completely ordered and decreases to near zero in a completely disordered state. Values of *Smol* less than 1.0 in the liquid crystalline state are a consequence of local chain tilting due to the presence of gauche conformers in the hydrocarbon chains. As temperature decreased, *Smol* increased in both summer and hibernating animals indicating a progressive increase in membrane acyl chain ordering. However, a divergence in the order parameter was evident at temperatures approaching the gel to liquid-crystalline phase transition temperature (as determined by DSC). At 270° K (-3° C) *Smol* was 29% higher in membranes from summer animals compared to hibernators (0.252 *vs* 0.196). The diverging order parameter profiles indicate that at any given temperature the ordering of membrane lipids in summer animals is greater, i.e., the membrane lipids are less fluid, and that the liquid crystalline-gel phase transition is lower in hibernating animals.

DPH Fluorescence polarization spectroscopy

The effect of temperature on the polarization of DPH incorporated into the inner mitochondrial membrane of summer-active and hibernating ground squirrels is shown in Fig. 4.5. Approximately 60 polarization/ temperature values were obtained for each preparation (n=9 for each state) and all values were pooled. At 4° C the polarization ratio was .344±0.006 (mean±S.E.) in summer animals and .337±0.004 in hibernators indicating that the average microviscosity of the hibernating membrane was lower, but the difference was not statistically significant. As temperature increased the polarization of the incident light increased, indicating an increasing angular excursion of the DPH probe within the membrane and thus indicating an increase in average fluidity with temperature. At 37° C the polarization ratio was higher in summer animals than in hibernators (.221±0.009 *vs* .212±0.007) indicating a less fluid lipid environment, however, again

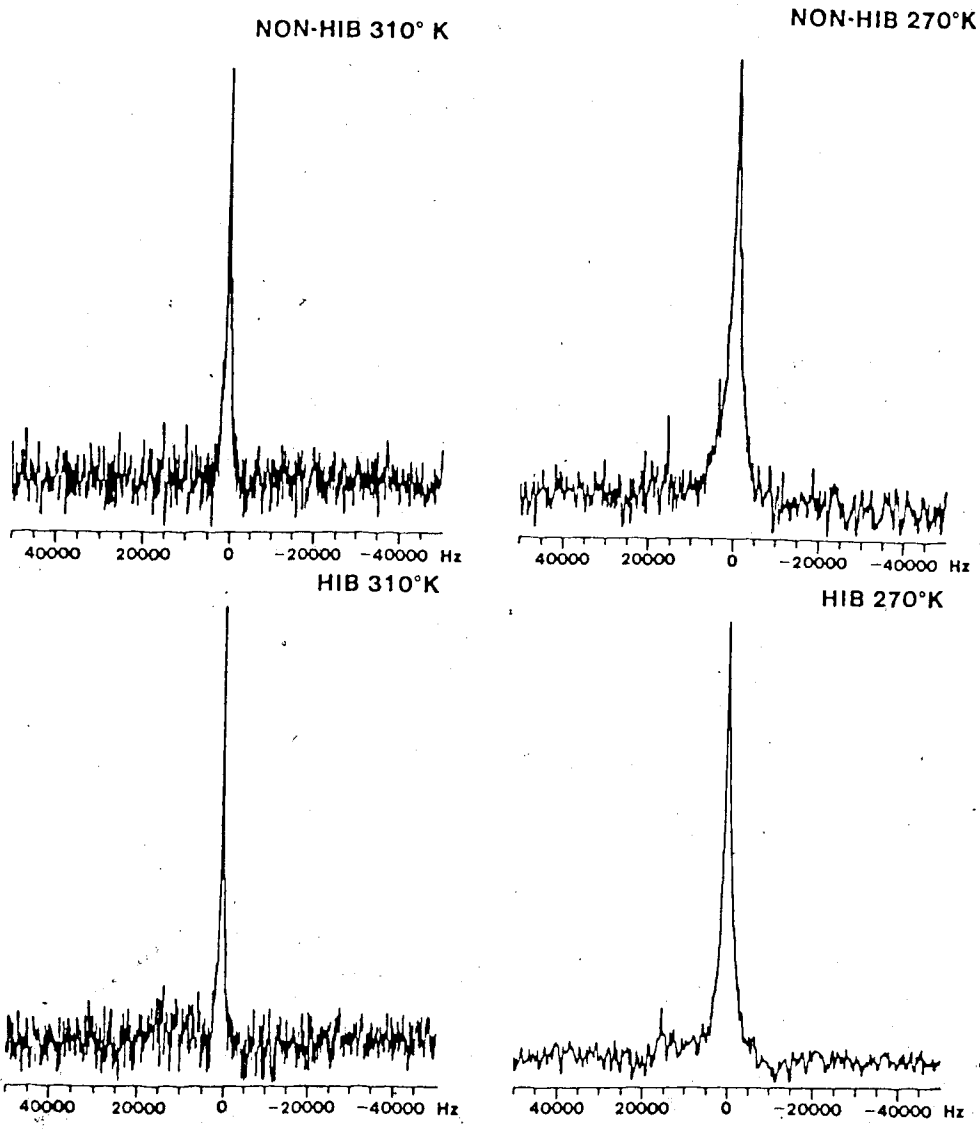


Figure 4.3. ^{19}F NMR line spectra from liver inner mitochondrial membranes from summer-active and hibernating ground squirrels.

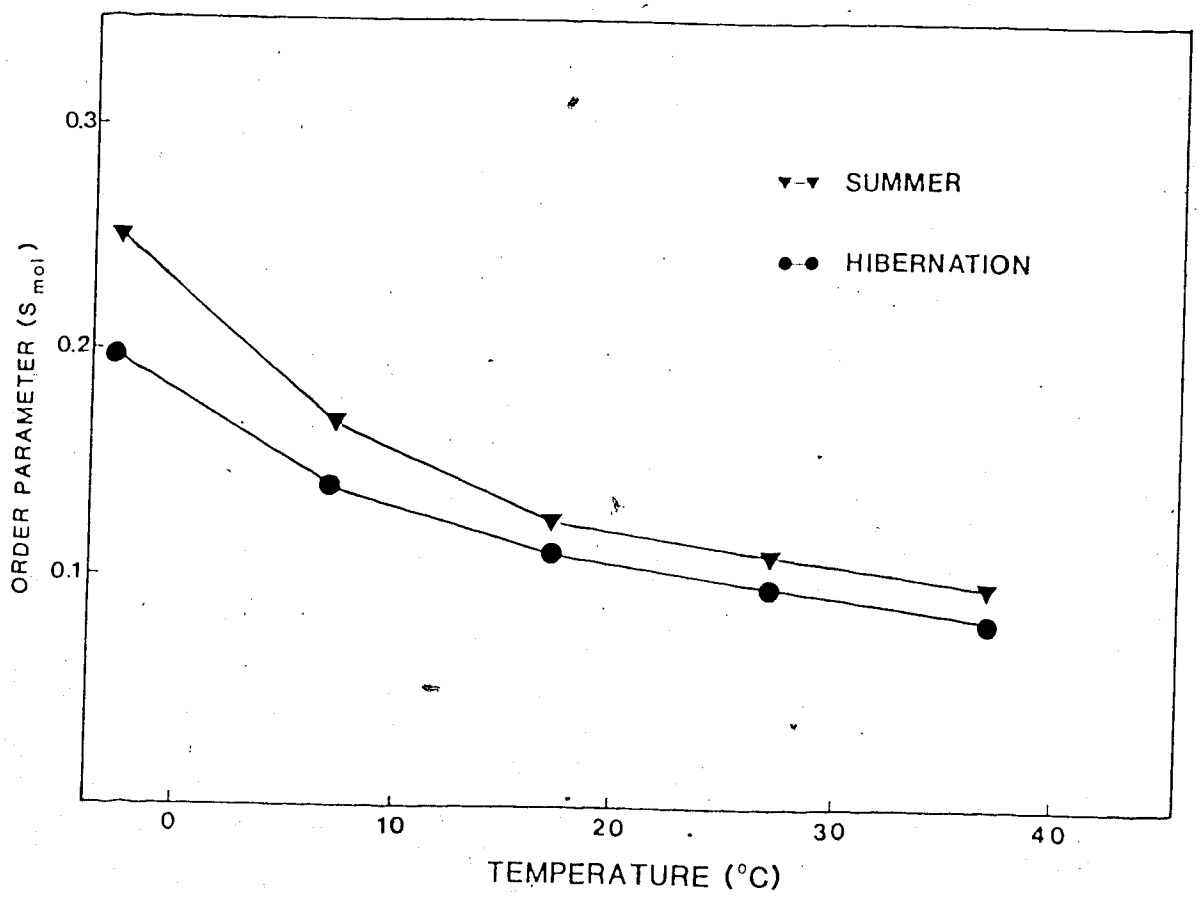


Figure 4.4. ^{19}F NMR orientational order parameter profiles of liver inner mitochondrial membranes from summer-active and hibernating ground squirrels.

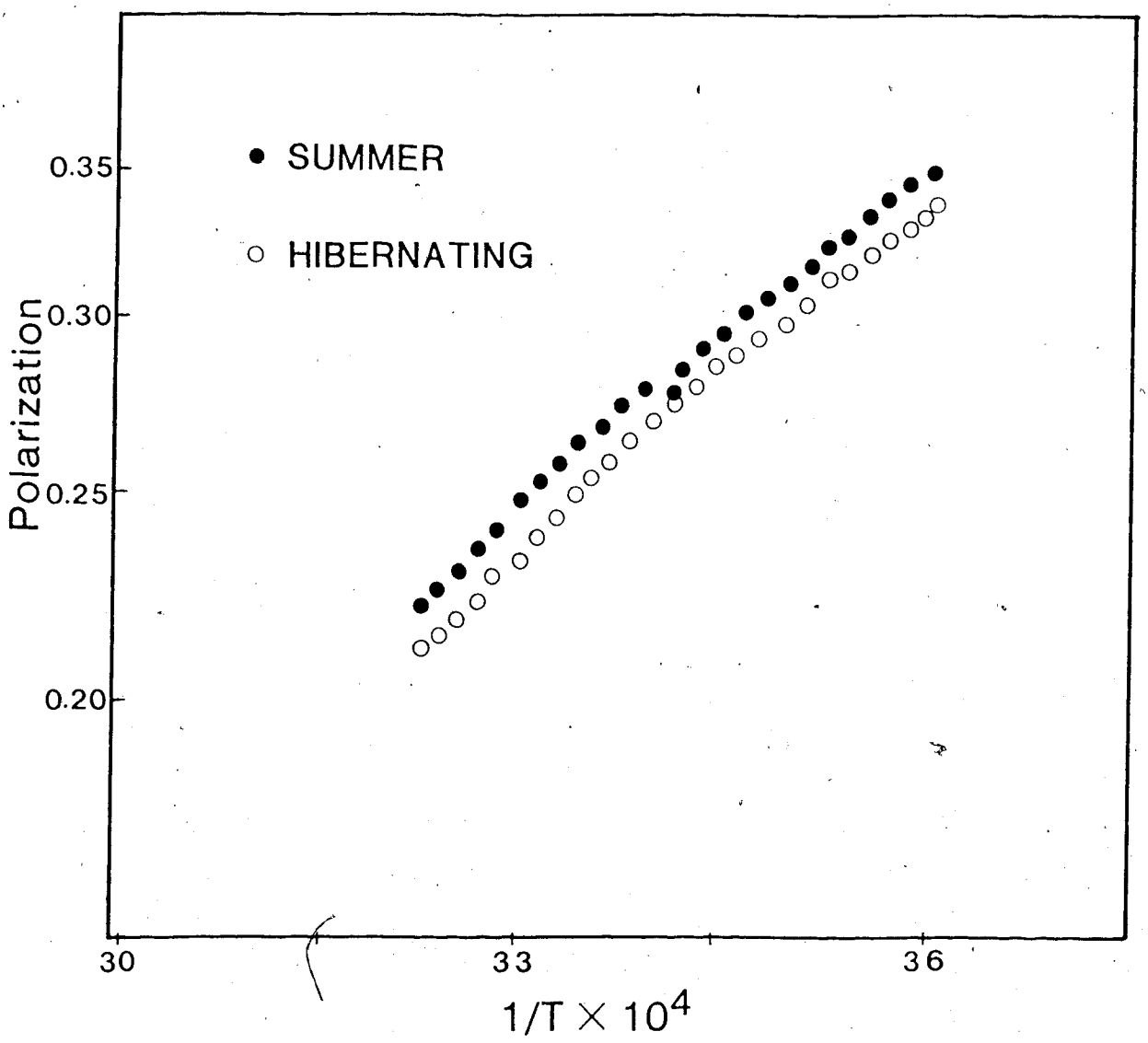
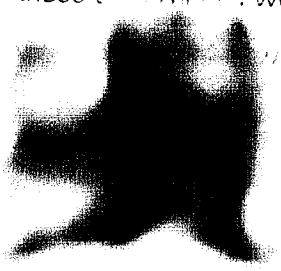


Figure 4.5. Arrhenius plot of DPH fluorescence polarization of liver inner mitochondrial membranes from summer-active and hibernating ground squirrels.

these [illegible] were not significant. The Arrhenius slopes of the pooled data points



[illegible]veen summer and hibernating animals, but, both lines were nearly linear

[illegible]f a sharp increase in lipid viscosity (*i.e.*, a phase transition)

D. Discussion

In a direct comparison between intact liver mitochondria and extracted mitochondrial lipids, Keith *et al*. (1975) observed non-linear Arrhenius plots of the rotational motion of the hydrocarbon spin label,2N12 in active animals but only linear plots in hibernators. The plots became non-linear near 20° C in intact mitochondria and near 23° C in lipids, suggesting a transition to a more ordered state below these temperatures. The Arrhenius plots were similar to those seen for succinoxidase activity in active ground squirrels (Raison and Lyons, 1971), suggesting a correlation between activity and the thermotropic behaviour of the mitochondrial membrane. More recently, Charnock *et al*. (1980) found corresponding shifts in transitions of myocardial membranes from summer-active and hibernating ground squirrels using DSC and 16NS ESR. The DSC-detected onset of the phase transition was lowered from 26° in summer active to 16° C in hibernators while ESR indicated transitions occurred at 29° and 18° C in summer and hibernating animals, respectively. However, there was no corresponding change in the behaviour of myocardial adenosinetriphosphatase activity in the same animals. In a study which compared Arrhenius plots of succinate-cytochrome *c* reductase activity and ESR of extracted lipids, Augee *et al*. (1984) found almost perfect correlation between enzymic activity and the thermal behaviour of the membrane lipids in liver mitochondria from ground squirrels in various phases of the hibernation cycle. Both assays also showed linear plots between 4 and 37° C in hibernators, and non-linear plots in animals not prepared for hibernation.

This study demonstrates that the non-linear Arrhenius plots of membrane-bound enzyme activity and ESR motion parameters seen in summer ground squirrels are probably not a consequence of the thermotropic behaviour of the bulk lipid phase. The gel-liquid crystalline phase transition of the inner mitochondrial membrane occurred near -2° C in summer-active animals, and even if this was corrected for a 6° C depression caused by 50% ethylene glycol (Steim *et al*., 1969) the transition is still at least 15-18° below the transition temperatures seen in enzyme activity and ESR signal. If the Arrhenius behaviour of enzyme activity and ESR were directly related to the bulk lipid phase properties we should only see a 3-4° C lowering of the break temperature in hibernators corresponding to our observed lowering of the gel-liquid crystalline phase transition temperature. This is

clearly not the case as Arrhenius plots were linear down to 4°C, the lowest temperature used.

The DSC data from all groups examined in this study are quite succinct but provide little information to explain the phase behaviour seen in membranes from other hibernating tissues and species. While we would anticipate the lipid domains of other types of membranes to vary, it is difficult to explain a gel-liquid crystalline conformational change seen at 16° C in myocardial membranes in hibernating ground squirrels (Charnock *et al.*, 1982), especially in tissue which has been demonstrated to be particularly cold tolerant in hibernating species (Burlington *et al.*, 1976). Even mitochondrial membranes from the rat, a species not capable of hibernation, show transitions near 0° C (Hackenbrock *et al.*, 1976), and essentially all phospholipids in mammalian membranes are considered to be in a fluid state above 5-10° C (Overath and Thilo, 1978). The calculation derived from the transition enthalpy that indicated no more than 2% of the myocardial membrane lipid is involved in the transition (Charnock *et al.*, 1980) is in contrast to the observation of Blazyk and Steim (1972) and others (Overath and Thilo, 1978) that 60-90% of membrane lipid takes part in a transition.

There is no apparent explanation for the large hysteresis seen between heating and cooling transitions in membranes from the hibernating and other winter groups examined in this study. At the relatively high scan rates necessary to observe phase transitions in a low lipid-to-protein ratio membrane, such as the inner mitochondrial membrane, instrumental heat lag may account for the observed hysteresis. However, it is not readily apparent why the difference between heating and cooling scans is only about 4° in summer animals compared to 11° in other hibernating states. One characteristic of lipid mixtures in biological membranes that has been observed, however, is that in the broad transition typical of the complex lipid composition of these membranes, the lipids of the co-existing fluid and ordered domains are different (Shimsick and McConnel, 1973). A change in membrane lipid composition between summer and hibernating states may result in distinct patterns of lateral separation and distribution of lipid species between the two states and exhibit different phase transition characteristics when going from a gel to liquid-crystalline conformation than from liquid crystalline to gel. A lower hydrocarbon chain order parameter detected by NMR in membranes from hibernators in this study supports the

lowering of the exothermic phase transition, and also demonstrates the absence of any transition above 0° in summer-active animals.

The fluidity probe 1,6-diphenyl-1,3,5-hexatriene is the most efficient of the fluorophores available for the hydrocarbon region of lipids (Shnitzky and Barenholz, 1978), partitioning equally into the liquid-crystalline and gel phases of membrane lipids. Using DPH Cossins and Wilkinson (1982) found no difference between synaptosomes from active and hibernating 13-lined ground squirrels whereas Montaudon *et al.* (1984) reported differences in brain microsomes and microsomal lipids in active and hibernating European hamsters. Critical examination of the latter data reveals, however, that the difference was quite small, in fact, it was even less than we have observed in ground squirrel inner mitochondrial membranes between the active and hibernating states. The Arrhenius curves for active and hibernating hamster microsomal membranes are nearly superimposable at temperatures approaching 2° C, the region in which we would expect significant differences if an increase in membrane fluidity is crucial for improved cold tolerance. From the existing fluorescence polarization data we would have to conclude that there is also no significant change in the average fluidity of the inner mitochondrial membrane during hibernation, at least not of the magnitude necessary to be termed a 'homeoviscous' adaptation (Cossins and Wilkinson, 1982). It should be noted, however, that there is some debate as to whether the apparent microviscosity measured by a fluoroprobe represents the bulk lipid phase or only lipid domains in the immediate vicinity of the probe (Hare *et al.*, 1979). The effect of proteins on microviscosity must also be taken into consideration in that proteins can increase microviscosity in membranes with low cholesterol to phospholipid ratios, such as mitochondria (Shnitzky and Barenholz, 1978), and in particular the inner membrane which has a low lipid-to-protein ratio.

In summary this study has demonstrated that a thermotropic transition occurs in the ordering of inner mitochondrial membrane lipids, and that the temperature of this transition is lowered in hibernating animals. ^{19}F NMR analysis of the inner membrane also indicates a lowering of the transition temperature and further suggests that acyl-chain ordering is lower in hibernating compared to summer animals over the entire physiological temperature range. When these results are taken together with the fluorescence polarization analysis, it is apparent that the fluidity of the bulk lipid phase of the inner

mitochondrial membrane is only slightly greater in the hibernating state than in the summer active state. It is significant however, that all three physical measurements of the thermotropic behaviour of the membrane show a distinct absence of a phase transition over the entire body temperature range (0-37°C) experienced by ground squirrels either in the hibernating or summer-active state.

V. SEASONAL CHANGES IN THE FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM LIVER INNER MITOCHONDRIAL MEMBRANE

A. Introduction

One of the unique aspects of mammalian hibernation is the continued functional integrity of cellular membranes at low physiological temperatures. From our knowledge of the analogous situation of thermal acclimation in poikilotherms (Hazel and Prosser, 1974), the possibility of the membrane lipid composition being a determinant of the capacity to hibernate has been an attractive hypothesis. The general trend in procaryotes and non-mammalian eucaryotes acclimated to low temperature is toward an increase in the proportion of tissue unsaturated fatty acids (Hazel and Prosser, 1974). The possibility exists that the fatty-acyl chain composition of membrane lipids may also play a role in mammalian hibernation by increasing membrane fluidity to compensate for the ordering effects of low temperature. Membrane fluidity is an imprecise concept which refers to the viscosity or molecular ordering of the membrane lipids (Quinn, 1981). While the fluid properties of a membrane are attributed solely to the lipid constituents, other factors such as membrane proteins, pH ,and ions such as Ca^{2+} can markedly affect fluidity(Kimelberg, 1976). In general the fatty-acyl residues influence the thermotropic properties of lipids depending on their chain length (Silvius *et al.*, 1979) and the presence of *cis* unsaturated double bonds (Barton and Gunstone, 1975). As chain length increases so does the temperature of the main phase transition ,and in synthetic phosphatidylcholines *cis*-unsaturated acyl chains show a lower transition temperature than do homologous saturated phosphatidylcholines having the same chain length.

Several comprehensive examinations of membrane lipid composition in hibernators have been documented and are summarized in reviews (e.g., see Aloia and Pengelley, 1979; Aloia, 1980). Taken as a whole, there does not appear to be a unifying pattern of fatty acid compositional changes in preparation for hibernation, with each tissue exhibiting a distinct distribution of fatty acids. This is not totally unexpected as there is a wide diversity of lipid composition between species and between tissues in the same species (Quinn and Chapman, 1979), with the individuality of lipids being expressed by differences in fatty acid composition. In brain microsomes from hibernating hamsters the overall trend

was toward an increase in mono- and di-unsaturates and a decrease in chain length among the polyunsaturates (Blaker and Moscatelli, 1978). The same variation was seen in erythrocytes from hibernating 13-lined ground squirrels, i.e., carbon chain reduction and increased unsaturation (Rotermund and Veltman, 1980). Platner *et al.*(1976) showed qualitatively similar changes in the fatty acid makeup of ground squirrel liver mitochondria, however, they reported very low levels of polyunsaturated fatty acids in both active and hibernating animals.

This study was undertaken in order to document seasonal changes in fatty acid composition of the inner mitochondrial membrane between the summer-active and hibernating states. A documentation of the fatty acid composition is necessary in order to provide a possible explanation for observed changes in the thermotropic behaviour of membrane-related functions and phase behaviour as detected by physical methods.

B. Materials and Methods

Animals

Richardson's ground squirrels were collected and kept as described in Chapter 2.

Isolation of inner mitochondrial membrane

Whole mitochondria were isolated as described in Chapter 2 and the inner mitochondrial membrane prepared as described in Chapter 4.

Extraction of total lipids

Total lipids were extracted from freshly prepared or lyophilized liver inner mitochondrial membrane preparations by the basic Folch extraction (Folch *et al.*, 1957) with minor modifications. All solvents were bubbled with N_2 before use and contained 0.05% butylated hydroxy toluene (BHT) as antioxidant. Membranes were dissolved in 5 volumes of methanol in 16 x 125 mm glass culture tubes containing glass beads and then 10 volumes of chloroform was added. Tubes were capped with a teflon-lined screw cap and the contents mixed vigorously for 60 sec on a *Vortex* mixer. The tubes were then centrifuged for 1 min to sediment the protein fraction and the solvent phase was filtered through polyester. The residue was resuspended in chloroform / methanol 2:1 (v / v) mixed, centrifuged and filtered again. The residue was resuspended in chloroform / methanol / 28% aqueous ammonia (7:1 ,v / v, saturated with NH_4). After centrifuging the extracts were pooled and evaporated under vacuum and N_2 at 5-10°C. The 28% aqueous ammonia was prepared fresh by bubbling NH_3 into double glass-distilled water.

Removal of non-lipid contaminants

Non-lipid contaminants were removed by chromatography on Sephadex G-25 (Pharmacia) as described by Wuthier (1966). Chloroform / methanol / water (8:4:3) were mixed and allowed to separate into two phases. Sephadex was made into a slurry with 100 ml of upper phase and equilibrated at least 3 hr before use. A column was prepared and topped with 1 cm of sea sand and then rinsed first with 10 column volumes of upper

phase and then 10 column volumes of lower phase. Total lipid extracts were dissolved in 2-3 ml of lower phase and applied to the column under a stream of N_2 then eluted with 10 column volumes of lower phase, the eluent was collected under N_2 and evaporated under vacuum and N_2.

Separation of phospholipid fraction

The phospholipid fraction of total lipid extracts was obtained by chromatography on silicic acid. A slurry of 2.5 gm of silicic acid (100-200 mesh, BioRad) in chloroform was poured into a 1.6 x 20 cm column and washed with 10 column volumes of chloroform. The total purified lipid extract was dissolved in 1-2 ml of chloroform and applied to the column under a stream of N_2 and neutral lipids eluted with 10 volumes of chloroform. The phospholipid fraction was eluted from the silicic acid with 10 volumes of methanol and evaporated under vacuum and N_2. Phospholipids were dissolved in chloroform / methanol (19: 1) containing 0.05% BHT and stored under N_2 in sealed ampuoles at -28°C.

Identification of phospholipid classes

The phospholipid class composition of the inner mitochondrial membrane was determined by two dimensional TLC on 250 *u*m Silica H plates (Applied Sciences). Plates were first activated by heating at 110°C for 1 hr, cooled in a chamber flushed with N_2 at 50% relative humidity and then spotted with an aliquot of phospholipid. A single spot containing 200-300 *u* g of phospholipid in chloroform was dried under N_2 and the plate developed in chloroform / methanol / 28% aqueous ammonia 65:25:5. The TLC solvent contained BHT and was degassed with N_2 prior to development. When the solvent front reached 1 cm below the upper edge of the plate, the plate was removed and dried under N_2 and then developed in a second solvent consisting of chloroform / acetone / methanol / acetate / water (3:4:1:1:0.5). After development in the second solvent the plate was dried under N_2 and sprayed with ANS. Phospholipid classes were located under U.V. and identified by comparison of relative retention times with known standards chromotographed under identical conditions.

Separation of phospholipid classes

Phospholipid classes were separated by isocratic high pressure liquid chromatography using the solvent system of Kaduce *et al.*(1983). A solvent ratio of 100:3:0.04 acetonitrile / methanol / H_2SO_4 was found to provide the best resolution of phospholipid peaks within a reasonable elution time. The solvent delivery system was a Waters Model 590 pump with programmable WISP sampler. Phospholipid classes were detected with a Waters Model 481 variable wavelength U.V. detector at 203 nm. Separation was attained with a 10*u* M Porasil column at a solvent flow rate of 2 ml per min. Peaks were recorded with a Linear recorder and phospholipids collected with a Gilson Model 280 fraction collector. Phospholipid peaks corresponding to cardiolipin, phosphatidylethanolamine and phosphatidylcholine were collected in 16 x 125 mm screw capped culture tubes under a stream of N_2.

Methylation of phospholipid fatty-acyl chains

Fatty acid methyl esters were derived from each phospholipid class and total phospholipids. Two ml of 14% boron trifluoride in methanol (Sigma) was added directly to each tube containing phospholipids in HPLC solvent, the tube flushed with N_2 and sealed with a teflon-lined cap. Tubes were heated at 110°C for 2 hr. After cooling 1 ml of water was added to each tube and the methyl esters extracted with 2 ml of hexane / diethylether 95:5 (v / v) containing 0.05% BHT. Each extract was purified by silicic acid chromatography, dried, and redissolved in double glass-distilled chloroform for GLC analysis.

Gas chromatography of fatty acid methyl esters

The fatty acyl composition of cardiolipin, phosphatidylcholine, phosphatidylethanolamine, and total phospholipids was determined by chromatography on a Varian Vista 401 gas chromatograph equipped with data station. A 6 ft glass column packed with Silar 10C with Gaschrom Q 100 / 120 as stationary phase (Applied Sciences) provided adequate separation of fatty acyl chains from C16 to C22 within 25 min. Elution temperature was programmed from 150 to 225°C at a rate of 3° per min. Methyl esters were detected with a flame ionization detector with detector and ionization chamber

temperatures set at 250°C. Nitrogen was used as carrier gas at a flow rate of 30 ml per min.

Each fatty acyl component was indentified by its retention time relative to stearate (18:0) and comparison to standards. Because the FID detector responds to the weight of each component analyzed, correction factors were calculated to determine the mol% of each fatty acyl constituent. Pure standards of each fatty acid of interest were precisely weighed before injection onto the column and the molar response calculated for each ester by dividing the area detected by the molar concentration, all other esters being relative to 18:0.

C. Results

A representative chromatogram illustrating the separation of the major fatty acids found in the inner mitochondrial membrane is shown in Fig. 5.1. Baseline separation of each fatty acid was achieved by temperature programming sample elution from 150 to 225°C at a rate of 3°C per min. Several other fatty acids were identified (20:1, 20:3, 22:4 and 24:1); however, in most cases they represented less than 0.5% of the total area chromatographed and were not quantified.

Total phospholipids

Virtually all of the fatty acids which comprise the inner mitochondrial membrane phospholipids changed between the summer-active and hibernating states (Table 5.1). The most significant increases during hibernation were seen in oleic (18:1) and linoleic acid (18:2) with slight increases in stearic (18:0) and arachidonic acid (20:4). There was a corresponding decrease in palmitic (16:0), palmitoleic (16:1), linolenic (18:3), docosapentaenoic (22:5), and docosahexaenoic acids (22:6). In general the fatty acid composition of the four remaining groups examined (aroused, cold- and warm-acclimated, and spring) were not significantly different from hibernating animals and are summarized in Table 5.1. Exceptions were the levels of 18:1, which were the same in cold-acclimated, spring and summer animals, and 22:5, which was the same in spring and summer animals.

An examination of the relative distribution of unsaturated double bonds revealed that the unsaturation index (UI) was higher in summer animals than in hibernators but this difference was not statistically significant (Table 5.2) The UI of the cold- and warm-acclimated and spring groups were the same as summer animals. The most consistant distribution of fatty acids occurred in the total saturates, 16:0 and 18:0, the levels of which were identical in all groups. The mono-unsaturated fatty acids, 16:1 and 18:1, were significantly higher in hibernating and aroused animals while the polyunsaturated fatty acids were lower. The most significant differences were in the (n-6) fatty acids (18:2 and 20:4), which were elevated in all groups relative to summer animals. Conversely, summer animals had the highest levels of (n-3) fatty acids (18:3, 22:5, and 22:6), although the levels of (n-3) in spring animals was almost as high.

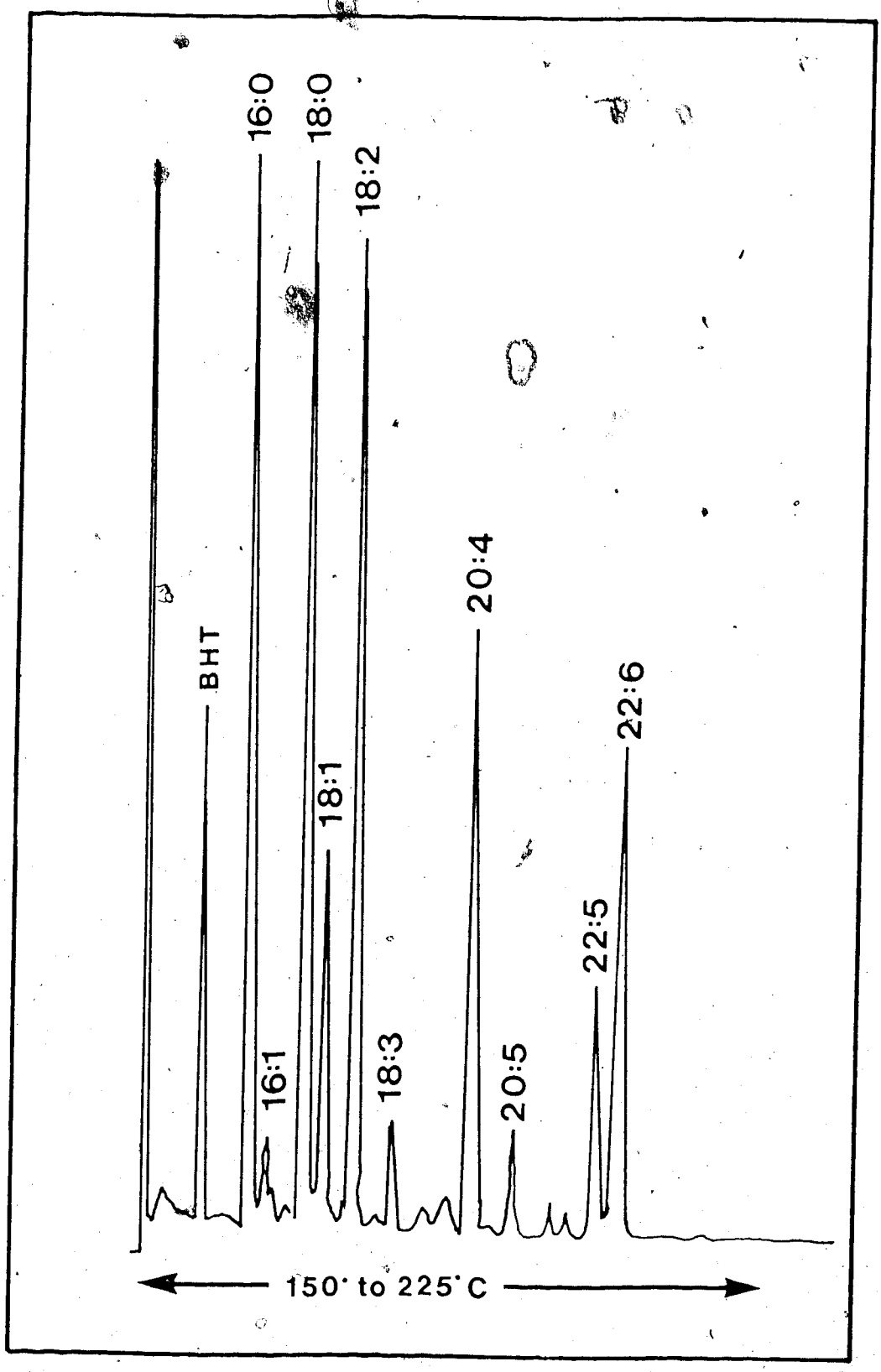


Figure 5.1. Representative gas chromatogram of fatty acid composition of phospholipids from liver inner mitochondrial membranes of the Richardson's ground squirrel.

Table 5.1. Fatty-acyl chain composition of total phospholipids from liver inner mitochondrial membrane from ground squirrels in different phases of hibernation cycle.

Phase	Fatty Acid Composition (mol%)*								
	16:0	*16:1*	*18:0*	*18:1*	*18:2*	*18:3*	*20:4*	*22:5*	*22:6*
Summer(11)	16.8±0.52	4.0±0.67	18.8±0.69	7.7±0.35	15.4±0.79	2.5±0.32	13.0±0.56	4.6±0.52	8.3±0.94
Hibernating(13)	14.4±0.49[1]	2.4±0.33[2]	21.0±0.57[1]	11.2±0.42[1]	22.0±0.79[1]	0.9±0.22[1]	15.7±0.92[2]	0.9±0.21[1]	5.8±0.39[1]
Aroused(5)	12.0±0.48[1]	2.0±0.29[2]	22.1±0.87[1]	11.9±0.65[1]	23.9±1.10[1]	0.3±0.04[1]	16.1±0.86[2]	0.4±0.04[1]	4.2±1.00[1]
Cold(7)	13.3±1.13[1]	1.6±0.46[2]	21.3±1.20[1]	8.5±0.31	24.7±1.76[1]	0.9±0.60[1]	15.0±0.96[2]	1.3±0.30[1]	7.6±0.32
Warm(5)	13.3±0.85[1]	1.5±0.39[2]	22.0±1.46[1]	10.5±0.92[1]	25.8±0.36[1]	0.5±0.12[1]	15.9±0.91[2]	1.2±0.42[1]	5.7±1.17[1]
Spring(4)	15.2±1.32.	1.6±0.07[2]	19.0±0.81	8.4±0.42	20.1±1.03[1]	2.9±0.54	13.6±0.55	4.2±0.66	6.7±0.39

*Values are means ± SE and represent a mean of 94% of the total area detected.

Statistically significant difference from summer group [1](P<0.001); [2](P<0.05).

Numbers in parentheses are numbers of individuals in each group

Table 5.2. Influence of hibernation state on proportion of unsaturated bonds in total phospholipids from inner mitochondrial membrane.

	Total Sat.	Total Mono.	Total Poly.	Total (n-6)	Total (n-3)	U.I.
Summer	35.6±0.61	10.1±0.51	26.1±1.39	28.4±1.04	15.4±1.35	179.2±7.29
Hibernating	35.1±0.77	13.7±0.63[1]	23.1±1.02[2]	37.7±1.31[1]	7.5±0.59[1]	163.3±5.02
Aroused	35.0±0.47	13.9±0.87[1]	23.1±2.60[2]	42.3±1.69[1]	4.6±1.46[1]	162.9±9.42
Cold	34.3±0.41	11.1±1.02	23.8±1.18[2]	39.7±0.57[1]	8.8±0.79[1]	174.3±3.13
Warm	35.4±1.05	11.9±0.98	23.5±0.79[2]	41.7±2.00[1]	7.4±1.15[1]	170.1±4.55
Spring	34.2±0.87	9.7±0.58	24.7±0.63	32.7±2.17[2]	13.8±0.88[2]	174.9±3.82

Values are means ± SE.

Total Sat. = Sum of saturated fatty acids.

Total Mono. = Sum of monounsaturated fatty acids.

Total Poly. = Sum of polyunsaturated fatty acids.

U.I. = Unsaturation index.

Statistically significant difference from summer group [1](P<0.001); [2](P<0.05).

Cardiolipin, phosphatidylethanolamine and phosphatidylcholine

The fatty acid composition of cardiolipin, phosphatidylethanolamine and phosphatidylcholine are summarized in Table 5.3. The same trend seen in the total phospholipid fraction was seen in phosphatidylcholine except that 20:4 decreased in hibernators. There was no difference in the levels of saturated fatty acids between summer-active and hibernating animals, however the total amount of saturates were higher in phosphatidylcholine than in the total phospholipid fraction. As in total phospholipids, the mono-unsaturated fatty acids increased and the polyunsaturates decreased in hibernation (Table 5.4). The (n-6) fatty acids were higher in hibernators and the (n-3) fatty acids lower and the unsaturation index was lower as well. Phosphatidylcholine had the lowest UI of the major phospholipids in both the summer-active and hibernating states and the index was sigificantly lower than the overall UI of the phospholipid fraction.

In the phosphatidylethanolamine fraction the largest increase occurred in 20:4 in hibernators and there was a corresponding decrease in 22:5 and 22:6. This was reflected in the ratio of (n-6) to (n-3) fatty acids which increased from 1.55 in summer-active animals to 5.1 in hibernators. Phosphatidylethanolamine had the highest levels of polyunsaturated fatty acids of the major lipid classes. There was virtually no difference in the UI between summer-active and hibernating animals.

The only major change in the cardiolipin fraction occurred in the (n-6) fatty acids, in particular 18:2, which increased 8 mol% in hibernators. Again the UI was lower in hibernating animals, but not significantly.

Table 5.3. Fatty-acyl chain composition of major phospholipids of liver inner mitochondrial membrane from active and hibernating ground squirrels.

Fatty Acid (mol%)*	Phosphatidylcholine		Phosphatidylethanolamine		Cardiolipin	
	Active	Hib.	Active	Hib.	Active	Hib
16:0	20.2±0.99	20.9±0.95	19.1±1.06	15.7±0.88[1]	15.2±1.06	14.5±1.48
16:1	2.8±0.77	1.4±0.58	1.71±0.25	2.0±0.32	10.0±1.19	5.1±0.63[1]
18:0	16.5±1.12	19.9±0.88[1]	27.4±1.01	32.4±1.90	9.3±0.62	7.6±0.58
18:1	8.9±0.91	12.1±0.45[2]	5.3±0.56	7.2±0.62[1]	11.2±1.74	17.5±1.35[1]
18:2	12.1±1.04	23.8±1.16[1]	5.6±0.39	9.3±0.76[1]	33.6±1.12	41.2±1.28[1]
18:3	3.4±1.02	1.1±0.28[3]	0.9±0.26	1.1±0.39	4.2±0.85	1.6±0.29[1]
20:4	12.4±1.30	9.0±0.68[3]	15.5±1.17	20.3±0.93[1]	2.9±0.65	2.2±0.43
22:5	4.1±0.89	TR	4.2±0.98	1.80±0.68[3]	2.3±0.32	2.2±0.43
22.6	8.9±1.02	3.0±0.38[1]	11.6±1.50	5.4±0.49[1]	2.3±0.75	2.1±0.38

*Values are means ± SE.

Statistically significant difference between active and hibernating groups: [1](p<0.001); [2](p<0.005); [3](p<0.05).

Table 5.4. Proportion of unsaturated bonds in major phospholipids from the inner mitochondrial membrane of active and hibernating ground squirrels.

	Active	Hibernating
Phosphatidylcholine		
Total Sat.	38.8±0.86	40.1±0.98
Total Mono.	13.0±0.97	16.4±1.05[3]
Total Poly.	13.9±1.31	6.9±0.59[1]
(n-6)	21.6±1.24	27.5±1.13[3]
(n-3)	4.5±0.34	3.9±0.41
U.I.	104.0±6.30	92.5±5.11
Phosphatidylethanolamine		
Total Sat.	43.2±1.40	47.9±1.40
Total Mono.	6.9±0.63	7.8±0.45
Total Poly.	25.9±1.88	27.2±1.41
(n-6)	21.2±1.21	30.6±1.54[1]
(n-3)	13.7±1.46	6.0±0.45[2]
U.I.	156.1±10.13	157.3±5.47
Cardiolipin		
Total Sat.	25.5±1.89	22.4±2.00
Total Mono.	25.9±1.62	26.1±1.40
Total Poly.	6.5±0.92	3.7±0.54[3]
(n-6)	36.1±1.23	43.1±1.97[1]
(n-3)	6.0±0.97	5.9±0.86
U.I.	162.8±6.72	145.9±7.90

Values are means ± SE.

Statistically significant difference from active group [1](P<0.001); [2](P<0.005); [3](P<0.05).

Determined by two-tailed Student's t-test.

D. Discussion

The overall trend in the transition from the summer-active to hibernating state was an increase in the ratio of (n-6) to (n-3) fatty acids, increasing from 1.84 in summer animals to 5.03 in hibernators. This combined with an increase in total mono- and di-unsaturates reflects a shift toward shorter-chain unsaturated fatty acids from polyunsaturated fatty acids. The mean chain length calculated from the total phospholipid fatty acids decreased from 16.8 to 16.2 in the transition from summer to hibernation. It is noteworthy that these changes occurred while the total levels of saturated fatty acids remained constant in all phases of hibernation indicating that the observed changes were a consequence of the interconversion of existing fatty acids and not the synthesis of new fatty acids. Goldman (1978) found increased liver stearoyl CoA desaturase activity in hibernating hamsters which could account for the increase in monenes consistently observed in liver membranes (Chaffee *et al.*, 1965; Platner *et al.*, 1976), and the inner mitochondrial membrane.

In a comparable study in which Platner *et al.* (1976) examined fatty acids in ground squirrel liver mitochondria, a trend toward an increase in monounsaturated fatty acids during hibernation was also evident, although the absolute levels of all fatty acids were different than in this study. This is not surprising in view of the fact that the lipid composition of the inner and outer mitochondrial membranes differ (Quinn and Chapman, 1980). A similar pattern was seen in the fatty acid composition of myocardial membranes from active and hibernating Richardson's ground squirrels but the magnitude of change was greater in the myocardial membranes than we have observed in the inner mitochondrial membrane and there was a significant decrease in saturated fatty acids in myocardial membranes of hibernators (Charnock *et al.*, 1980).

The fatty acid compositions of the major phospholipids were remarkably different from each other and each class showed a different profile for the hibernating state. Phosphatidylethanolamine showed the greatest increase in (n-6) fatty acids during hibernation, 9.4 mol%, and a 7.7 mol% decrease in (n-3) fatty acids. The reduction in (n-3) fatty acids represented nearly 50% of the total level of polyenoic acids. These changes would be expected to increase the fluidity of the membrane because of the lower melting point of (n-6) fatty acids compared to (n-3) (Sober, 1970). Similar increases in the ratio of

(n-6) to (n-3) fatty acids were observed in brain microsomes from hibernating hamsters (Goldman, 1975; Blaker and Moscatelli, 1978). Because phosphatidylethanolamine comprises nearly 40 percent of the phospholipid composition of the inner mitochondrial membrane (Colbeau *et al.*, 1971), these alterations could influence the overall fluidity of the membrane. Phosphatidylcholine also showed an increase in the ratio of (n-6) to (n-3) fatty acids during hibernation again at the expense of polyunsaturated fatty acids. Support for the conversion from poly to monounsaturates comes from the fact that animals cannot desaturate fatty acids in the (n-6)(n-3) positions of the hydrocarbon chains of monoenoic fatty acids (Fulco, 1974). Phosphatidylcholine also makes up nearly 40 percent of the inner mitochondrial membrane lipids and can thus also contribute significantly to a change in overall fluidity. Cardiolipin is almost exclusively found in the inner mitochondrial membrane and is characteristically highly unsaturated, the dominant fatty acid being 18:2 (Ioannou and Golding, 1979). In hibernating ground squirrels 18:1 and 18:2 comprised 60% of the total fatty acids in the cardiolipin fraction, an increase of 11 mol% over the summer-active state. This is compatible with the essential role of cardiolipin in the maintenance of the integrity of the inner mitochondrial membrane (Stuhne-Sekalek and Stanacev, 1977) and as a structural component of the respiratory chain (Ioannou and Golding, 1979). Cytochrome *c* oxidase has a catalytic requirement for cardiolipin with enzymatic activity being dependent on two tightly bound cardiolipin molecules (Fry and Green, 1980). Because it is inextricably linked to the electron transport chain, it would appear to be advantageous for cardiolipin to increase or at least maintain its fluid nature during hibernation.

Although some tissues in hibernators show an increase in total fatty acid unsaturation, that does not appear to be the case in the inner mitochondrial membrane. In whole brain (Aloia, 1979) and brain microsomal phospholipids (Goldman, 1975) the unsaturation indices increased between the active and hibernating states but in heart and liver (Aloia and Pengelley, 1979; Platner *et al.*, 1976) membrane fatty acid unsaturation either remained the same or decreased during hibernation. However, when the relative contribution of individual phospholipid classes was taken into consideration, the total unsaturation index of whole brain was determined not to be significantly different during hibernation (Aloia, 1980). Our observations, therefore, strengthen the argument that the

degree of unsaturation of membrane bulk lipids is not crucial for hibernation. Indeed, Coolbear *et al.* (1983) have shown that increasing the unsaturation of a single acyl chain in a phospholipid does not necessarily lower the gel to liquid-crystalline transition temperature of the lipid and may not increase fluidity. While the introduction of *cis* double bonds at carbons 9 and 12 of C*18* lecithins lowered the transition temperature dramatically, the further introduction of double bonds actually increased the transition temperature slightly (Coolbear *et al.*, 1983). Therefore it would appear that it is the location of the double bonds and not necessarily the total numbe r of double bonds (*i.e.*,unsaturation) which may influence fluidity.

It is clear that aroused animals are able to retain the distribution of fatty acids seen in hibernation and indicates that the fluid state of the membrane is compatible with a wide range of body temperatures. The same conclusion can be drawn from the fatty acid composition in cold- and warm-acclimated animals which have not experienced torpor. These animals appear to be physiologically ready for hibernation with respect to the phase state of the inner mitochondrial membrane while still in euthermia. It is also apparent in the spring animals that when hibernation has ended, a reversion back to the summer state of fatty acid composition is initiated.

In summary, the proportion of monounsaturated fatty acids increased in liver inner mitochondrial membranes during hibernation as did the (n-6) series of fatty acids comprised mainly of linoleic and arachidonic acid (18:2 and 20:4). The overall composition of the membrane phospholipids reflected the contribution of the major phospholipid constituents, cardiolipin, phosphatidylethanolamine and phosphatidylcholine. The pattern of fatty acid distribution which evolves in preparation for hibernation appears to be one that is capable of increasing the fluidity of the membrane. This conclusion is based on the influence of *cis*-unsaturated fatty acids on the thermotropic behaviour of membranes, *i.e.*, *cis*-unsaturated fatty acids effectively lower the gel to liquid-crystalline phase transition temperature, presumably because the *cis* double bond prevents the close hexagonal packing required to form the ordered gel-phase (Barton and Gunstone, 1975). These authors also demonstrated that the most effective location of the *cis* double bond is near the middle of the hydrocarbon chain. This is precisely what we have seen in the membranes from hibernators, a shift toward *cis*-unsaturated fatty acids with the *cis* bond

predominantly near the middle of the hydrocarbon chain.

When unsaturated fatty acid-supplemented diets were fed to rats, an increase in the (n-6):(n-3) ratio was observed in mitochondrial membranes, with a corresponding decrease in *Ea* for Arrhenius plots for succinate-cytochrome *c* reductase activity (McMurchie *et al.*, 1983). By our own estimates a significant change in *Ea* was brought about by only a 45% increase in the (n-6) to (n-3) ratio in the rat membrane, thus a nearly 3-fold increase in the (n-6) to (n-3) ratio in hibernators would appear to be substantial. The observations of McMurchie *et al*. linking membrane fatty acid composition with Arrhenius behaviour of membrane enzymes may have relevance to seasonal changes seen in ground squirrel mitochondrial lipids in conjunction with hibernation.

GENERAL DISCUSSION

The task set out at the beginning of this study was to determine if significant adaptations occur in the structure and function of liver mitochondria in preparation for hibernation. The ultimate goal was to determine if a unique characteristic of the inner mitochondrial membrane, either the thermodynamics of respiration or the thermotropic properties of the lipid bilayer brought about by alterations in its lipid constituents, enhances the cold tolerance of mitochondria in a typical hibernator. For the purposes of comparing the various physiological states, cold tolerance is defined as exhibiting an Arrhenius constant for mitochondrial respiration which is invariant with temperature over the range of body temperatures encountered in a hibernation bout, typically 4-37°C.

Critical to this study was the necessity to demonstrate if a change in cold tolerance or sensitivity occurs in liver mitochondrial respiration between the summer-active and hibernating states, as suggested by Raison and Lyons (1971) and Roberts *et al.* (1972). Willis *et al.* (1981) and Willis (1982) have critically evaluated the evidence presented to date and have concluded that liver mitochondria from hibernators are probably not unique in their ability to function at low temperatures. However, the conclusive evidence incorporating a comprehensive examination of the bioenergetics, physical characteristics and lipid composition of liver mitochondria has yet to be presented.

In Chapter 1 it was clearly shown that both oxidative phosphorylation, and the activity of the respiratory chain in the absence of phosphorylation, show distinct differences between the active and hibernating states. While other authours have shown little or no differences in cold tolerance in mitochondria in other species (eg. Liu *et al.*, 1969; Roberts *et al.*, 1972), the apparent changes in the temperature dependence of respiration below a critical temperature in active animals in this study implies the opposite situation is true in Columbian and Richardson's ground squirrels. The rigorous evaluation of the Arrhenius behaviour with the use of ADP-stimulated or steady-state respiration with A-23187 precludes any ambiguous intepretation of the results. A question that must be addressed, however, is the validity of the thermodynamic information we can extract from the Arrhenius plots. Bagnall and Wolfe (1982) have argued that the slope of non-linear Arrhenius plots are not proportionally related to the activation energy, and the

implication that Arrhenius breaks in complex membrane systems delineate only the midpoint in the energetics of a transition (Silvius and McElhaney, 1981) caution against over-interpretation of the significance of Arrhenius plots.

The underlying causes of Arrhenius non-linearity are difficult to determine experimentally. The apparent energy of activation, Ea, which we calculate from the simplified Arrhenius equation is only an approximation of the true enthalpy of activation for an enzymic reaction and is a kinetic rather than a thermodynamic parameter (Klein, 1982). Non-linear Arrhenius plots can arise from either kinetic or thermodynamic consequences, the former usually being the result of a change in the rate-limiting step in a muti-step catenary enzyme reaction (Han, 1972). It is important to realize that non-linear plots can occur due to changes in the rate of reaction where the equilibrium enthalpy may remain the same, but a change in entropy occurs. A change in entropy for simple reactions can be related to randomness or order-disorder phenomena (Klein, 1982) and for this reason non-linear Arrhenius plots may be associated with membrane transition effects. Therefore, while Arrhenius plots of mitochondrial activity were unequivocally linear during hibernation, we would have to assume that in active animals the departure from linearity at some point along the temperature axis means that one or more of the factors contributing to the Arrhenius equation has changed. The temperature dependence of the reaction geometry, activation entropy or activation enthalpy would have to change dramatically to produce the change in slope seen in active animals (McElhaney, 1982).

A clear interpretation of the shift from a non-linear to linear plot is impeded by the depression of respiratory activity during hibernation, which implies a kinetic factor superimposed on the thermodynamics of the process. The release of this inhibition during the arousal process indicates that the lower rates seen in hibernation are probably not the result of fewer functional respiratory units, but may reflect a decrease in the number of active functional units or a decrease in efficacy in each unit. It is difficult to perceive the synthesis of more functional units during the short time span of an arousal bout immediately following a period of metabolic depression and leading into another period of metabolic depression when hibernation resumes. The linear Arrhenius plots in hibernating animals is very convincing, however, and conform to our definition that an increased cold tolerance occurs in the hibernating state.

Chapter 2 delineated the seasonal aspect of hibernation and revealed that hibernation is not a simple, two-phase physiological shift. Again we are confident that the trends indicated by Arrhenius plots are valid by virtue of the correspondence between three simultaneous functions of the electron transport chain, *i.e.*, proton efflux, Ca^{2+} uptake and oxygen consumption. The findings in this study were corroborated by an independent study by Augee *et al.*(1984), in which the temperature dependence of succinate-cytochrome *c* reductase activity and ESR spin motion of lipid extracts showed nearly identical Arrhenius behaviour to the ones we have presented in Chapter 2. It should be emphasized that the former results were obtained from the same mitochondrial preparations as the latter. A caveat that must be registered against the results of Augee *et al.*, however, is the criticism of interpreting ESR spin label motion as a measurement of a thermotropic transition in the membrane lipids. DSC analysis of the inner mitochondrial membranes showed the bulk lipids to be in a predominantly liquid-crystalline state near 0°C in all phases of the hibernation cycle (Chapter 4).

While there are few other seasonally-oriented studies to compare ours to, Cremel *et al.*(1979) did examine the correlation between the seasonal variation in lipid composition with intramitochondrial protein movement in hamster liver mitochonria. There was a distinct seasonal variation in membrane fatty acids and an implied alteration in membrane fluidity; however, these did not correlate well with Arrhenius breaks in intermembranal protein movement. In contrast to our observed seasonal differences in Arrhenius plots, another mammalian hibernator, the bent-winged bat, showed linear Arrhenius plots for succinate-cytochrome *c* reductase activity or ESR order parameter year round (Geiser *et al.*, 1984). These hibernators can exhibit torpor at all times during the year suggesting a consistent relationship between linear Arrhenius plots and the ability to tolerate low body temperature.

Our examination of the bioenergetics of mitochondria in summer and hibernating animals produced some unexpected results. It was clearly demonstrated that the mechanism of energy-linked Ca^{2+} uptake is maintained during hibernation. The adaptive significance of continued Ca^{2+} regulation is self evident, especially in view of the fact that tissue Ca^{2+} levels increase during hibernation (Ferren *et al.*, 1971). Under conditions of relative intracellular overload, the mitochondria become the predominant site of Ca^{2+}

buffering (Carafoli, 1982), and in the absence of fine control over Ca^{2+} uptake and release, Ca^{2+} can induce membrane permeability changes and a collapse of the inner mitochondrial membrane potential (Beatrice *et al.*, 1980), effectively halting respiration. The interesting observation was the maintenance of H^+ / Ca^{2+} and Ca^{2+} / O stoichiometries in active animals at all temperatures. The stoichiometries persist despite an apparent change in the temperature dependence of respiratory activity. This suggests that the permeability properties of the inner mitochondrial membrane are maintained at low temperatures, even in summer animals, and that the non-linear plots are the consequence of changes in enzyme conformation or lipid-protein interactions, but not the result of thermally induced damage to the membrane. It should be stressed, however, that the mitochondria from the active animals were only exposed to low temperature for 2-3 hr (preparation and assay time) compared to several days of low body temperature in the hibernating animals. It is possible that long-term cold exposure may lead to membrane permeability changes in mitochondria from summer-active animals.

To this point we had accumulated convincing evidence that the inner mitochondrial membrane is well adapted for functioning at low temperatures during the hibernating state. Physical measurements of the thermotropic behaviour of the membrane revealed that there was an increase in the mobility of membrane lipids during hibernation and that there was a concomitant lowering of the gel to liquid-crystalline phase transition temperature. Neither DSC analysis, ^{19}F NMR spectroscopy or fluorescence spectroscopy detected a thermotropic phase transition above 0°C in mitochondria from any phase of the hibernation cycle. Further, the increase in overall fluidity of the bulk lipid phase between the summer and hibernating states was very limited. This suggests two possibilities, 1) that the differences seen in mitochondrial activity are not a consequence of a phase transition in the bulk lipid environment, and 2) the inner mitochondrial membrane is sufficiently "fluid" to support continued function at all times of the year. Clearly, we can discount a phase transition as being responsible for non-linear Arrhenius plots of mitochondrial respiration as "breaks" in Arrhenius plots of membrane activity did not correspond to T*max* (by DSC), nor did they indicate the upper boundary of the transition (completion temperature). The second possibilty is not as easily resolved. Even though the increases in fluidity did not appear to be great, virtually all animals examined in the hibernating state exhibited linear

Arrhenius plots and a lowered gel-liquid crystalline phase transition temperature. Conceivably, an increase in membrane fluidity in the hibernating state may be an adaptation for efficient energy conservation by the cell. Such a proposal is not without its merits, as increased viscosity implies kinetic constraints (Lands, 1981), which would have to be compensated for to maintain a constant membrane environment. Again, though the lipid domains of other membranes may vary, non-linear Arrhenius plots seen in rabbit muscle sarcoplasmic reticulum could not be altered by changing the fluidity of the membrane with cholesterol (Madden and Quinn, 1979), suggesting that thermotropic changes in protein arrangement are not necessarily affected by changes in fluidity. Thomas and Hidalgo (1978) showed that non-linear Arrhenius plots of sarcoplasmic reticulum Ca^{2+}-ATPase were associated with a decrease in the rotational motion of the protein and that ATPase activity correlated better with protein mobility than lipid fluidity. Finally, East *et al.* (1984) have recently demonstrated that there was no correlation between ESR-detected order parameters and ATPase activity in (Ca^{2+}-Mg^{2+})-ATPase from sarcoplasmic reticulum when the enzyme was reconstituted with phospholipids of varying unsaturation.

An alternative hypothesis to a change in fluidity of the bulk lipid pool is the existence of a "boundary layer" or "lipid annulus" (eg. Houslay and Stanley, 1980). Such a scheme envisions an immediate lipid ring surrounding an integral protein, which provides an interface between the protein and the bulk lipid pool. The greater interaction between the protein and annulus would result in phase transition behaviour different from the bulk lipid phase. The existence of annular lipids is predicated on ESR-detected "fluid" and "immobile" phospholipid environments in protein-lipid dispersions. The concept of boundary-layer lipids with long-lived shells of lipid controlling enzyme activity has been discounted by NMR analysis (Chapman *et al.*, 1979). It would appear that ESR data showing an immobile component is merely the consequence of the rapid motions of bulk phase lipids in the ESR time scale. NMR, which generally samples longer time scales, and recent ESR data has shown that a "one site" rather than a "two site" (fluid and immobilized lipid) is a more appropriate interpretation (Meirovitch *et al.*, 1984).

The final portion of this study was intended to provide evidence for a lipid-mediated increase in cold tolerance in hibernators. An examination of the literature makes it clear that a single unifying concept in the strategy of lipid modification during

hibernation may be an elusive goal and was not the aim of the present study. Indeed, many changes seen in the transition to the hibernating state may not be related to cold tolerance at all. For example, levels of phosphatidylcholine in kidney increase significantly in hibernating ground squirrels, but this may only be a consequence of temperature inhibition of the group transfer reaction which forms sphingomyelin from phosphatidylcholine and would thus increase tissue levels of that phospholipid (Aloia and Pengelley, 1979). We have presented evidence that changes in fatty acid composition in the inner mitochondrial membrane are consistent with the increase in fluidity as detected by DSC and NMR. Aloia (1980) concluded that the existing information on the role of fatty acids in hibernation created considerable doubt as to the contribution of the fatty acids to membrane fluidity. He based his conclusions strictly on the total unsaturation of the membrane phospholipids which does not appear to change in most hibernating tissues examined. We suggest that unsaturation is indeed not the major determinant of increased fluidity of the membrane, but rather it is the conversion to mono- and di-unsaturated and (n-6) fatty acids which may be of prime importance. By critically re-evaluating the fatty acid composition data available (Goldman, 1975; Platner *et al.*, 1976; Blaker and Moscatelli, 1979; Aloia, 1979), it is apparent that this is indeed the case as all of these studies revealed the same trend. Again, we cannot ignore the possibility that seasonal changes in fatty acid composition may not necessarily be related to the fluidity of the membrane. Membranes in general are known to be assymetrical with regard to lipid distribution within the bilayer (Bretscher, 1972) and may be highly compartmented (Aloia and Rouser, 1975). Alterations in fatty acid composition during hibernation may be dictated more by the requirements of specific regions of the membrane or the activity of certain enzymes rather than the overall fluidity of the bilayer (Aloia, 1980).

In summary, this study has brought together functional, structural and physical properties of the energy-transducing inner mitochondrial membrane of two mammalian hibernators. It has been demonstrated that there are seasonal variations in the cold tolerance of liver mitochondrial respiration-linked activity in both the Columbian and Richardson's ground squirrel, the apparent energy of activation being temperature independent in the hibernating state. While mitochondrial activity is apparently less cold tolerant in summer-active animals, the exact nature of the temperature sensitivity and its

energetic consequences have not been resolved.

The fluidity of the inner mitochondrial membrane appears to be enhanced by a seasonal alteration in the fatty acid composition in preparation for hibernation. But, even though the changes in the thermotropic behaviour of the membrane coincide with temporal changes in activity, no direct correlation between the two phenomena has been established. Therefore, though the adaptive significance of large scale lipid-mediated fluidity changes may be attractive, it is probably more reasonable to expect only limited changes in one parameter in the overall process of adaptation for hibernation.

At this point it does not appear possible to extrapolate our findings to other membrane types, tissues or species. While mitochondria from all sources share basic functions, there is enough diversity among different tissues to prevent a generalized application of our observations to tissues such as heart or kidney. On the other hand, virtually all mitochondria participate in energy production, to a greater or lesser extent, and it would be unreasonable to conclude that not all mitochondria are cold tolerant in the hibernating state manifested in the Columbian and Richardson's ground squirrel. Obviously the energy requirements of some cells are minimal during hibernation, but the energy demands during arousal and inter-torpor bouts dictates that the integrity of all mitochondria must be maintained.

To the ground squirrel as a whole, the source of energy is a prime consideration during the fasting hibernation season. As might be expected the predominant metabolic pathway during hibernation and arousal is lipid metabolism, the loci of fatty acid oxidation residing in the mitochondrion. We suggest that the conditions we have reported in liver mitochondria necessarily encompass maintaining the integrity of this function as well. Clearly the ability to function over a wide temperature range represents a significant adaptation in mitochondria. What remains to be resolved is whether the characteristics of the inner mitochondrial membrane we have documented are unique to hibernators in general, or only to the two species we have investigated.

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