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Effects of Induced Cold Torpor and Hypoxia on Cardiac Mitochondrial Cytochrome Oxidase Activity, Phospholipid Content and Ultrastructure in the Fresh Water Turtle, Pseudemys Scripta

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Animals were divided into three groups: room temperature controls, and experimentals which were acclimated to 5°C cold or cold-hypoxia for two weeks. Arrhenius plots of COX activity of controls had an activation energy \( E_a \) of 10.77 kcal mole\(^{-1}\) for the temperature range of 5-35°C, whereas those of both cold-acclimated groups showed discontinuities at 30°C. \( E_a \)'s of turtles adapted to cold alone were 10.99 kcal mole\(^{-1}\) (5-30°C) and 5.155 kcal mole\(^{-1}\) (30-35°C), while those exposed to cold-hypoxia were 11.25 kcal mole\(^{-1}\) (5-30°C) and 2.14 kcal mole\(^{-1}\) (30-35°C). Lubrol-treated mitochondria exhibited no such "breaks". These results suggest that COX activity is viscotropically regulated during cold acclimation. Mitochondrial levels of phosphatidyl ethanolamine (PE) decreased significantly in both cold-acclimated groups \((P < 0.001)\), whereas diphosphatidyl glycerol (DPG) increased significantly in animals acclimated to cold alone \((P < 0.005)\). Morphometric analysis showed ischemic, significant increases of mitochondrial area of both cold-acclimated groups.
(P < 0.001), with no alterations of shape.
Effects of Induced Cold Torpor and Hypoxia on Cardiac Mitochondrial Cytochrome Oxidase Activity, Phospholipid Content and Ultrastructure in the Fresh Water Turtle, *Pseudemys scripta*.

by

George Demos

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

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1992
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF RELATED LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>11</td>
</tr>
<tr>
<td>Protein Determination</td>
<td>12</td>
</tr>
<tr>
<td>Cytochrome c and Cytochrome Oxidase</td>
<td>12</td>
</tr>
<tr>
<td>Phospholipid Extraction</td>
<td>14</td>
</tr>
<tr>
<td>Phospholipid Determination</td>
<td>15</td>
</tr>
<tr>
<td>Lipid Detection and Quantitation of Phospholipid</td>
<td>16</td>
</tr>
<tr>
<td>Transmission Electron Microscopy</td>
<td>17</td>
</tr>
<tr>
<td>RESULTS</td>
<td>20</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>43</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>51</td>
</tr>
</tbody>
</table>


LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Arrhenius plots for COX activities of control animals maintained for two weeks at room temperature</td>
<td>25</td>
</tr>
<tr>
<td>2. The effect of cold acclimation upon COX activities of animals allowed to surface freely</td>
<td>27</td>
</tr>
<tr>
<td>3. The effect of hypoxic, cold acclimation upon COX activities of turtles which were forcibly submerged</td>
<td>29</td>
</tr>
<tr>
<td>4a. Representative micrograph of cardiac muscle fibers of a turtle maintained at room temperature. Magnification X 10,000</td>
<td>31</td>
</tr>
<tr>
<td>4b. Representative micrograph of cardiac muscle fibers of a turtle maintained at room temperature. Magnification X 30,000</td>
<td>33</td>
</tr>
<tr>
<td>5a. Representative micrograph of cardiac muscle fibers of a turtle which was cold acclimated for two weeks, and allowed to surface freely Magnification X 10,000</td>
<td>35</td>
</tr>
<tr>
<td>5b. Representative micrograph of cardiac fibers of a cold, normoxic turtle. Magnification X 30,000</td>
<td>37</td>
</tr>
<tr>
<td>6a. Representative areas of cardiac muscle fibers from turtles acclimated to two weeks of cold-hypoxia. Magnification X 10,000</td>
<td>39</td>
</tr>
<tr>
<td>6b. Cardiac fibers of cold-hypoxic turtles, at a magnification X 30,000</td>
<td>41</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mole percentage of mitochondrial phospholipids</td>
<td>21</td>
</tr>
<tr>
<td>2. Activation energies of COX from turtles with three distinct acclimation histories</td>
<td>23</td>
</tr>
<tr>
<td>3. Results of morphometric analysis of mitochondrial areas and shape factors, from animals described in the previous tables</td>
<td>24</td>
</tr>
</tbody>
</table>
INTRODUCTION

One of the dominant themes in biology is the concept of adaptation, or the modification of an organism's characteristics which allow for survival and reproduction in a particular environment. To this end, all living systems require the preservation of the structural integrity of their macromolecules and the maintenance of mechanisms for regulation of metabolic rates in varying environments. Further, adequate supplies of energy and of metabolites must be available for the synthesis of glycogen, lipids, and nucleic acids. (Hochachka and Somero, 1984).

Reptiles, like other groups of animals, respond in a number of ways to seasonal changes inflicted during winter months in a temperate climate. Some species remain active while others retreat to underground or underwater sites and begin a period of dormancy or cold torpor (Gatten, 1987). The freshwater turtle is a typical poikilotherm which experiences the latter. Through a reduction in its overall metabolic rate at decreased temperatures, viability is increased during submergence (Musacchia, 1959). The cardiovascular system plays a pivotal role in its survival during this time. Documented changes include a decrease in heart rate to as low
as eight beats per hour at 3° C (Jackson and Ultsch, 1982),
conservation of cardiac glycogen at the expense of that within
the liver (Crawford, 1984), increased cardiac anaerobic
glycolysis during submergence (Privitera and Beall, 1969),
and, following an initial discharge, maintenance of a stable
level of cardiac high energy organophosphates (Makris and
Rotermund, 1989).

Submerged turtles will occasionally surface during
moderate winter days, which necessitates a switch from
anaerobic to aerobic metabolic pathways (Gatten, 1987).
Cytochrome oxidase (COX), associated with the latter pathway,
is a major enzyme found in the mitochondrial respiratory
chain. COX, an integral protein located within the inner
mitochondrial membranes, catalyzes the electron transfer from
reduced cytochrome c to oxygen.

The question of how cardiac cytochrome oxidase
functions during thermal adaptation at temperatures below 15° C
is the focus of the present investigation. The kinetics of
COX and the mitochondrial phospholipid population following
acclimation to low temperature and/or hypoxia will be
measured. Also, electron micrographs of cardiac tissue will
be used to determine whether ultrastructural differences occur
in mitochondrial size and/or shape.
The ability of an organism to adapt to a dynamic environment plays a major role in its viability and survival. Gravity, water, nutrients, oxygen, temperature, as well as mechanical, magnetic and electrical stimuli function in concert as physical properties of an organism's environment (Prosser, 1986). A significant change in any of these physical properties creates a situation which requires a response from the organism. The adaptive procedures available to each particular organism influence where it can live and whether or not it will be able to survive.

Temperature is one of the most important and constantly changing environmental parameters, particularly in temperate climates of the world. Residents must adapt to the temperature extremes of hot summers and frigid winters. Their adaptation strategies can range from behavioral thermoregulation, viz. by seeking shade or moving underground, or underwater, to the tremendous biochemical and biophysical changes involved with hibernation during winter. The latter changes are designated as capacity and resistance adaptation.

Capacity adaptation refers to properties which allow for biological functions to continue over a 'normally' varying
environment. These include alterations of enzymes and/or lipids in order to maintain a stable or constant energy output, or membrane alterations which allow for maintenance of ionic and electrical gradients. Resistance adaptations occur at environmental extremes and may involve extensive biochemical changes, genetically predetermined, which allow animals to withstand lower or higher temperatures than those at which activity is optimal. For example, certain polar fish synthesize glycoproteins which act as antifreeze, lowering the freezing point of body fluids to allow for survival at very low temperatures (Prosser, 1986).

Hibernation is a resistance type of adaptation. The term generally means the passing of winter in a torpid or lethargic state. This definition, in its broadest sense, encompasses both poikilotherms (e.g. reptiles, amphibians, fish, insects) and homeotherms (mammals and birds) (Gatten, 1987). Reptiles, amphibians, and fish rely on heat from the environment to achieve and maintain warm internal body temperatures. When winter arrives and temperatures drop, poikilotherms experience hypothermia. In contrast, many scientists restrict the term 'hibernation' exclusively to a select group of mammals and birds which survive cold under a narrower range of conditions than reptiles and amphibians (Lyman, et al., 1982). For example, mammals and birds maintain a high steady body temperature throughout life, except during winter hibernation, which may have been
triggered by a combination of cold and lack of food (Hochachka & Somero, 1984). Once in the state of hibernation, homeothermic body temperature can drop to near 0° C (Spurrier & Dawe, 1973). Small mammals, such as woodchucks, enter hibernation and remain cold for about a week at a time, arousing for as long as a day. Other small mammals (termed 'feeding hibernators') arouse weekly and feed voraciously on stored food for about a day. Larger mammals, such as bears, remain dormant for up to seven months and do not eat, defecate, or urinate (Hochachka & Somero, 1984). The freshwater turtle is a typical poikilotherm. During the winter months it retreats to ponds and lakes and begins a period of underwater torpor, or dormancy, which may be interrupted by occasional surfacing during the warmer winter days (Gatten, 1987). Torpor is an intermediate type of hibernation in which body temperature declines markedly (Lyman, et al., 1982). The cardiovascular system plays a critical role in the survival of the turtle at such times. Like the vertebrate heart, the heart of the freshwater turtle is generally highly aerobic. However, it possesses a number of additional properties which allow for continued function during cold and hypoxia or anoxia. For example, glycogen reserves in the turtle myocardium are considerably greater than those of the rat. Under identical conditions of hypoxia, the turtle heart can develop 43% of prehypoxia tension in contrast to the rat heart which falls to less than 10% (Bing
et al., 1972). During submersion, turtles experience a reduction in overall metabolic rate, which contributes significantly to their increased viability at low temperatures (Musacchia, 1959).

Early in winter turtles utilize lipids for energy. As winter progresses and lakes freeze over, oxygen supplies become depleted and catabolism gradually is switched from lipids to carbohydrates via anaerobic glycolysis. Hepatic glycogen is utilized first while cardiac glycogen is conserved (Crawford, 1984). Glycogenolysis occurs via phosphorolysis by the enzyme glycogen phosphorylase. Of special interest is that the percentage of active phosphorylase in the turtle heart is eight times that of the mammalian heart; presumably the large quantity of active enzyme facilitates the initiation of glycolysis during hypoxia (McNeill et al., 1971). Also, unlike mammals, turtle phosphofructokinase (PFK) is regulated by phosphocreatine (PC). This high energy phosphagen reserve, after an initial discharge of ATP in turtle myocardia, is maintained at a stable level during extended cold and/or cold hypoxia; this would further sustain anaerobic glycolysis (Makris & Rotermund, 1989).

During hypoxic cold torpor, pyruvate is converted to lactate via lactate dehydrogenase (LDH), regenerating NAD⁺ and allowing glycolysis to continue. Under laboratory conditions, lactate levels have been found to increase by over four times in cold-hypoxic turtles (Privitera & Beall, 1969). Obviously,
the buffering capacity of overwintering turtles must be tremendous. Since $\text{HCO}_3^-$ is only partially effective, the balance of the buffering is thought to be accomplished by changes in 'strong ions'. Strong ions are those derived from strong electrolytes that completely dissociate in water solutions. $\text{Cl}^-$ decreases while $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ both increase (Jackson & Ultsch, 1982). However, field studies have shown that overwintering turtles do not accumulate the enormous quantities of lactate found in laboratory studies. It seems that slight amounts of oxygen are sufficient to keep lactate at a reduced level. Recently it was determined that *Chrysemys picta* has two major hemoglobin variants termed S and D. The S form has a much higher affinity for oxygen than does the D, and is the predominant form found in turtles in Minnesota and Wisconsin (*C. p. bellii*); it facilitates oxygenation of tissues in the cold. The D form was found in turtles from the delta region of Louisiana (*C. p. dorsalis*) (Ultsch, G., 1989).

In a comparative study, turtle cardiac LDH has been reported to be similar in molecular properties to that of rat muscle LDH. Cardiac LDH of turtles is composed of M type subunits which have a high affinity for pyruvate. This permits more efficient function under anaerobic conditions than the H type of mammalian hearts. Kinetic studies have shown that the turtle cardiac LDH has twice the affinity for substrate at $15^\circ\text{C}$ as it does at $25^\circ\text{C}$ (Beall & Privitera, 1973). Electrophoretic studies of isoenzymes indicated that
no new quantitative or qualitative changes of LDH are needed for survival under conditions of prolonged cold exposure or cold submergence (Beall & Privitera, 1973).

At temperature extremes, maintenance of the integrity of cellular membranes is extremely critical for survival. A number of biological processes are intricately involved with the plasma membrane. These include hormone recognition sites (DeRobertis et al., 1975), trans-membrane movement of ions (Pringle & Chapman, 1981) and viscotropic regulation (Kimmelberg & Papahadjopoulos, 1974). Since membranes consist primarily of a phospholipid bilayer, along with associated integral and peripheral proteins, it is important to understand what changes the phospholipid population experiences during temperature fluctuations. The key adaptation is maintaining membrane fluidity. This is usually accomplished by increasing unsaturation of the acyl groups (Cossins, 1977; Rotermund & Veltman, 1981), or formation of lysophosphatides (Aloia et al., 1974; Rotermund & Veltman, 1981), both of which serve to increase membrane disorder at low temperatures. This maintains fluidity which allows functional integrity of lipid dependent enzymes (Lyons & Raison, 1970; Raison et al., 1971; Raison & Lyons, 1971; Kimmelberg & Papahadjopoulos, 1974).

With respect to aerobic pathways that may be operating during moderate winter days, cytochrome oxidase (COX) is a major enzyme found in the respiratory chain. Cytochrome
oxidase is located within inner mitochondrial membranes and catalyzes the electron transfer from reduced cytochrome c to oxygen. COX has been demonstrated to be functionally dependent on its lipid environment (Sandermann, 1977). Diphosphatidyl glycerol (DPG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) are the typical, intrinsically associated lipids which affect its activity (Seelig & Seelig, 1984; Yu, Gwak & Yu, 1985). DPG is essential for maximal activity of COX and is the only phospholipid that cannot be replaced or substituted for by exogenous phospholipids without loss of activity (Robinson et al., 1980).

In the 1880's Arrhenius showed that only a small fraction of the total population of molecules possessed enough energy to react at certain temperatures. Increasing the temperature led to marked increases in the size of the reactive population (Hochachka & Somero, 1984). Arrhenius plots (Log $V_{max}$ vs the reciprocal of $1/T$ absolute) have been used to assess thermotropic alterations of protein structure, or temperature induced changes in substrate binding kinetics (Morris & Clark, 1981) over a thermal spectrum (Lyons & Raison, 1970; Raison et al., 1971; Raison & Lyons, 1971; Kimelberg & Papahadjopoulos, 1974; Cossins, 1977). The energy of activation ($E_a$) may be calculated from the slope of these plots, as well as temperature transitions of enzymatic activity. Such plots have shown that membrane bound ATPases of turtle cardiac mitochondria undergo conformational changes around 30° C,
thereby preventing active centers from properly aligning with ATP (Rotermund & Privitera, 1972). It is important to note discontinuities of the slopes may be produced also by lipid transitions (viscotypic changes) upon membrane bound enzymes (Charnock, 1978).

Investigations of ultrastructural modification have shown increases in the volume density of red muscle mitochondria of cold acclimated goldfish. The enlargements were attributed to an increase in size of mitochondria, as opposed to an increase in their numbers (Tyler & Sidell, 1984). Cardiac tissue of hibernating ground squirrels exhibits increases in the number of lipid droplets adjacent to mitochondria (Burlington, et al., 1972), along with increases in the number of mitochondria; there were no changes in mitochondrial size (Moreland, 1961).

The present research examines the effects of cold adaptation upon the kinetics of COX and the mitochondrial phospholipid population, in aquatic turtles exposed to cold or cold hypoxia. Electron micrographs will determine whether ultrastructural differences accompany functional changes. The purpose is to present a more coherent picture of structure/function relationships which permit survival of the species in the cold of a temperate environment.
MATERIALS AND METHODS

Female turtles (*Pseudemys scripta*), 8-10 inches in carapace length, were obtained from Konz Scientific Co., Oshkosh, Wisconsin, and maintained in the laboratory for a minimum of two weeks prior to experimentation. Subsequently they were divided into control and experimental groups. Twelve control animals were maintained at room temperature and fed liver *ad libitum*. They remain 'normoxic', having access to air and maintaining 'normal' plasma oxygen levels as defined by Ultsch (1989). Twenty four experimental animals were divided into two groups of twelve and placed into a Hotpack® Environmental Chamber at 4°C. Hypoxic animals were force-submerged under stainless steel grids for the two week period, while the remaining ones were allowed to surface freely. The latter group of turtles are considered cold-normoxic (Ultsch, 1989). All groups of animals were maintained under a photoperiod of 12:12 hr (light:dark). Animals experiencing cold torpor do not feed.

At the appropriate time, turtles from each group were killed by decapitation and their carapaces opened to expose their hearts. Ventricles were excised, immediately washed twice in chilled 0.25 M sucrose-Tris HCl, pH 7.4, blotted on
bibulous paper and weighed. Mitochondria were isolated using the procedure of Mersmann and Privitera (1964). A 10% homogenate (w/v) was prepared in sucrose-Tris HCl-EDTA, pH 7.4. The homogenate was centrifuged at 800 X g for 20 minutes at 5° C in a refrigerated Sorvall® RC2-B superspeed centrifuge. The supernatant was removed and placed onto ice while the nuclear pellet was resuspended in isotonic sucrose-Tris HCl-EDTA and re-centrifuged for 15 minutes. Both supernatants were then combined and centrifuged at 8000 X g for 20 minutes. The resulting mitochondrial pellet was resuspended in sucrose-Tris HCl, pH 7.4, and centrifuged for 15 minutes. The final mitochondrial pellet was resuspended in a volume of isotonic sucrose-Tris HCl equal to the weight of the original ventricle. This constituted the 'mitochondrial suspension' from which phospholipids were extracted, cytochrome oxidase (COX) activity was determined, and protein determinations were performed.

Protein Determination

Mitochondrial protein was determined by the method of Lowry et al., 1951. Protein content was adjusted to 0.35 mg/ml for COX determinations, and 3 mg/ml for phospholipid assays.

Cytochrome c and Cytochrome Oxidase

Cytochrome oxidase was assayed colorimetrically, using
a freshly prepared 1% solution of cytochrome c Type III (Sigma Chemical Co.) in a 0.01 M phosphate buffer, pH 7.0. This solution had been previously reduced using a minimum of sodium thiosulfate (Bergmeyer, 1983), which was subsequently removed by passage through a QS 2B Sephadex column (Isolab Inc. Akron, Ohio). Assays were performed either with or without a detergent enhancement procedure described by Rafael (1983). The detergent treated mitochondria were pre-treated with a 1% Lubrol PX solution (a non-ionic detergent consisting of ethylene oxide condensates of fatty-acid alcohols), which dissolves away the outer mitochondrial membrane. A volume of 0.1 ml Lubrol was added to 0.9 ml mitochondrial suspension. For optimal activity the organization of COX complexes was maintained with large excesses of nonionic detergent, which has been shown to disrupt the phospholipid-protein interaction without completely depleting COX complexes of lipid (Robinson and Capaldi 1977). Robinson et al. (1980) showed that two or three diphosphatidyl glycerol molecules were essential for maximal activity of the complex.

Two one ml cuvettes with a 10 mm light path were used for the COX assay (Wharton and Tzagoloff, 1967). The blank cuvette contained 0.1 ml of 0.1 M potassium phosphate buffer, pH 7.0 (temperature adjusted), 0.07 ml cytochrome c, 0.83 ml distilled, deionized water, and 0.01 ml potassium ferricyanide (0.1 M). The reaction was initiated in the second cuvette by the addition of 0.01 ml of the mitochondrial suspension
(either Lubrol treated or untreated) instead of the potassium ferricyanide. Prior to experimentation, a control had been performed to determine the amount of endogenous cytochrome c present in the mitochondria. The changing absorbances of all COX reactions were read at 550 nm at 5°C, 15°C, 25°C, 30°C, and 35°C on a Bausch & Lomb Spectronic 21 UVDR specially equipped with a thermal jacket. A GCA Precision Scientific refrigeration unit was used to maintain all temperature within 0.1°C. Absorbance values were converted to mg reduced cytochrome c based upon a standard curve using known amounts of cytochrome c. Enzyme activity was expressed in terms of nanomoles reduced cytochrome c/mg protein/minute ± SEM.

Phospholipid Extraction

All solvents used for extraction or two dimensional thin layer chromatography (2D-TLC) contained 50 mg/l butylated hydroxy-toluene (BHT) as an anti-oxidant. After mitochondrial isolation and protein determination, extraction was performed immediately according to the method of Kates (1972).

One ml of the mitochondrial extract (containing 3 mg protein) was placed into a 15 ml glass stoppered centrifuge tube containing 3.75 ml methanol-chloroform (2:1 v/v). The mixture was shaken intermittently for one hour and centrifuged at low speed in an International Clinical Centrifuge for five minutes. The supernatant was collected and transferred to
another centrifuge tube and the residue was re-extracted with 4.75 ml methanol-chloroform-water (2:1:0.8 v/v). After centrifugation for five minutes, both supernatants were combined and diluted with 2.5 ml each of chloroform and water and re-centrifuged for five minutes. The lower chloroform phase was withdrawn and diluted with an equal volume of benzene. The test tube was placed into a 30°C water bath and the contents were brought to dryness under a stream of nitrogen. The residue was immediately dissolved in 1 ml of chloroform and sealed under nitrogen and stored at -20°C until use. Phospholipids stored under these conditions are stable for up to two years (Kates, 1972).

**Phospholipid Determination**

Utilizing Analtech's uniplates (silica gel H, no binder, with 7.5% magnesium acetate precoated at 25 mm thickness), two dimensional thin layer chromatography was used to determine the mitochondrial phospholipid population (Supelco, 1971; Kates, 1972). Values were compared against those of standard phospholipids obtained from Sigma Chemical Co., St Louis, MO. The plates were activated for 30 minutes in a forced draft oven at 120°C and allowed to cool for 5 minutes. A volume of 200 microliters of extracted phospholipids was spotted at the lower right corner of each plate 2.5 cm from each edge. Spotted plates were then placed into solvent chambers (12" X 4" X 10" glass containers with
glass lids). Two chambers were used for two dimensional chromatography. The first chamber contained 500 ml chloroform-methanol-ammonium hydroxide (65:25:5, v/v) and the second contained 500 ml chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5 v/v). Filter paper was placed against the back of each chamber and left for 1 hour to allow for chamber equilibration. Spotted plates were then lowered gently into the first chamber and left until the solvent front reached 4-5 cm from the top. The plates were removed to a drying chamber, at room temperature, for 10 minutes under a stream of dry nitrogen. The plates were then rotated 90° clockwise and placed into the second chamber and left for 1 hour, or until the solvent front reached 4-5 cm from the top. The plates were removed and dried under nitrogen. Finally, a volume of 200 microliters of phospholipid was spotted on the upper right hand corner. This was known as the 'total' spot and was used to estimate the percent of total phospholipid recovered.

Lipid Detection and Quantitation of Phospholipid

The plates were developed under a hood by spraying each plate lightly with sulfuric acid dichromate (0.6% K₂Cr₂O₆ in 55% H₂SO₄) and placed into the forced draft oven at 120°C for 30 minutes. Phospholipids were detectable as charred spots, which were identified by comparison with standards. Standards used were phosphatidylethanolamine (PE), phosphatidylcholine
(PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), cardiolipin (DPG), lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). All charred spots, including the 'total' spot, were carefully scraped and placed into marked test tubes. An unspotted area was also scraped (about the size of an average spot) to be used as a blank. To each test tube 0.9 ml of 72% perchloric acid was added and the tubes were placed onto a hot plate at 160°C for 20 minutes. After acid digestion, 5 ml of water was added to each tube followed by one ml of ammonium molybdate solution (2.5%). After vortexing, one ml of freshly prepared ascorbic acid (10%) and one ml of water were added. The contents were again vortexed and placed into a boiling water bath for 5 minutes. The test tubes were cooled, centrifuged at low speed, and the clear supernatant transferred to cuvettes where absorbances were read at 820 nm on a Bausch & Lomb Spectronic 21. The absorbance values were converted to micrograms phosphorus by means of a factor derived from values obtained with known amounts of KH₂PO₄ standard (Kates, 1972). Results were expressed as mole % ± SEM.

A two tailed Student's t test was used for statistical analysis of all COX and phospholipid data.
Transmission Electron Microscopy

Tissue for electron microscopy was obtained immediately prior to excision of the heart from all three groups of animals. Upon opening of the pericardium, a transverse cut was made approximately one cm from the apex of the ventricle. This permitted the removal of small pieces of tissue (measuring approximately 10 mm in length and 5 mm in width) which were immediately placed into cold Karnovsky's fixative [2% (w/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde in cold 0.2 M sodium cacodylate buffer, pH 7.4]. Samples remained in the fixative for at least two hours, after which they were removed and cut into smaller pieces measuring approximately one mm³. The samples were then washed three times, 20 minutes each, in sodium cacodylate buffer (0.2M, pH 7.4) before 2 hours of post-fixation in 2% osmium tetroxide. The postfixied tissue was also washed in the sodium cacodylate buffer three times for 20 minutes.

The tissue samples were dehydrated using a graded series of acetone dilutions. Each step required 20 minutes, starting with a 30% dilution followed by 50%, 75%, 95%, and three times in 100%. The embedding process was initiated by placing the dehydrated samples into a 1:1 acetone/epon dilution overnight on a rotator. Subsequently they were placed into a 1:3 acetone/epon dilution for two hours followed by a second night of polymerization of epon, under a vacuum. This latter step was accomplished by placing the individual samples into molds,
covering them with epon, and incubating them overnight in an oven at 60°C. The molds were then removed and the samples were trimmed and sectioned on an LKB 2188 Ultratome. Thin sections were collected onto 300 mesh copper grids and stained in uranyl acetate and lead citrate. Grids were allowed to dry for two hours before being viewed on the JEOL JEM-1200 EX electron microscope. Magnifications selected for viewing were 4,000 X, 12,000 X, 15,000 X, and 30,000 X. Mitochondrial size and shape were measured morphometrically, with micrographs at a magnification of 12,000 X using the Bioquant II image analysis system. The perimeters of 150 mitochondria were measured in each group, with the orientation of the mitochondria being random relative to the longitudinal axes of the myofibers. Area was measured in \( \mu m^2 \), and shape was determined from a numerical scale where 0 is a straight line and 1 is a circle. Data were analyzed using Student's t test.
RESULTS

As is indicated in Table 1, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and diphosphatidylglycerol (DPG) were the major phospholipid species in turtle cardiac mitochondria. There were no significant differences in PC content across the three groups. In contrast, there were significant decreases in PE of 8% in the cold-normoxic (P< 0.05) and of 18% in the cold-hypoxic groups (P< 0.001). DPG increased by 16% in the cold-normoxic group (P< 0.005). Significant changes in the minor lipid species occurred in the cold-hypoxic group including a 33% increase in sphingomyelin (Sph) (P< 0.02) and a decrease in lysophosphatidylethanolamine (LPE) of 43% (P< 0.001).

Figures 1, 2, and 3 depict Arrhenius plots of COX activity from control, cold-normoxic, and cold-hypoxic populations, respectively. Curvilinearity occurs between 25° to 30° C in the plots of untreated mitochondria of cold-normoxic and cold-hypoxic animals. Comparison of the elevations of the plots shows a significant increase in the cold-normoxic (P< 0.001) group, vs. the control group, with no significant change in the cold-hypoxic group. Table 2 shows the activation energies (Ea) of COX calculated using the
### Table 1. Mole Percentage of Mitochondrial Phospholipids ± SEM

<table>
<thead>
<tr>
<th>Phospholipids&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Controls</th>
<th>Cold-acclimated</th>
<th>Hypoxic Cold-acclimated</th>
<th>P&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>40.53 ± 3.14</td>
<td>37.15 ± 2.81</td>
<td>33.27 ± 4.53</td>
<td>.05</td>
<td>.001</td>
</tr>
<tr>
<td>PC</td>
<td>38.47 ± 1.45</td>
<td>39.87 ± 2.17</td>
<td>41.64 ± 5.75</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DPG</td>
<td>14.27 ± 2.21</td>
<td>17.07 ± 2.08</td>
<td>16.50 ± 4.68</td>
<td>.005</td>
<td>NS</td>
</tr>
<tr>
<td>PS</td>
<td>2.73 ± 1.31</td>
<td>1.91 ± 1.28</td>
<td>2.41 ± 0.57</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PI</td>
<td>2.14 ± 1.45</td>
<td>1.68 ± 0.88</td>
<td>2.48 ± 0.93</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sph</td>
<td>2.06 ± 0.77</td>
<td>1.94 ± 1.03</td>
<td>3.07 ± 1.03</td>
<td>NS</td>
<td>.02</td>
</tr>
<tr>
<td>LPE</td>
<td>1.22 ± 0.41</td>
<td>1.26 ± 0.82</td>
<td>0.69 ± 0.22</td>
<td>NS</td>
<td>.001</td>
</tr>
<tr>
<td>% Recovery</td>
<td>98.9 ± 4.0</td>
<td>98.2 ± 2.7</td>
<td>98.1 ± 2.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> PE = phosphatidylethanolamine, PC = phosphatidylcholine, DPG = diphosphatidylglycerol (cardiolipin), PS = phosphatidylserine, Sph = sphingomyelin, PI = phosphatidylinositol, LPE = lysophosphatidylethanolamine

<sup>b</sup> Probability of significant difference
- 1. Difference between control (room temperature) and cold-acclimated groups
- 2. Difference between control and hypoxic, cold-acclimated groups
Arrhenius equation. There was no significant difference between the $E_a$ of the control population with/without the Lubrol detergent treatment. However, the COX activity following the Lubrol treatment had a significantly decreased $E_a$ in the cold-normoxic group ($P<0.01$). Further, significant decreases occurred in the $E_a$ in the Lubrol treated mitochondria of the cold-normoxic and cold-hypoxic groups compared to the COX activity of mitochondria without the Lubrol treatment ($P<0.001$ and 0.005, respectively).

Figures 4a, 4b, 5a, 5b, and 6a, 6b are transmission electron micrographs showing numerous mitochondria between myofibrils of cardiac muscle. Figures 4a and 4b were taken from the control turtles showing muscle striations, with mitochondria scattered among abundant glycogen reserves. Enlarged mitochondria containing dense granules were evident in the cardiac muscle from cold-normoxic and cold-hypoxic animals (Figures 5a, 5b and 6a, 6b, respectively). Table 3 illustrates the results of morphometric measurements of mitochondrial perimeters for size and shape. Mitochondrial area showed significant increases of 214% in the cold-normoxic and 278% in the cold-hypoxic groups ($P<0.001$) while shape was slightly spherical in all groups. Glycogen, although not measured, appeared to be somewhat depleted in the cold-normoxic and hypoxic groups. Dense inclusion bodies were seen within mitochondria of the cold-normoxic and hypoxic groups. No other morphological changes were immediately apparent.
Table 2. Activation Energies of COX activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control(17)</th>
<th>Normoxic, Cold-Acclimated(22)</th>
<th>Hypoxic, Cold-Acclimated(17)</th>
<th>( p_1^c )</th>
<th>( p_2^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Lubrol</td>
<td>10,770</td>
<td>10,990</td>
<td>11,250</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Lubrol</td>
<td>10,195</td>
<td>8,000</td>
<td>8,910</td>
<td>.01</td>
<td>NS</td>
</tr>
<tr>
<td>Lubrol VS No Lubrol</td>
<td>NS</td>
<td>.001</td>
<td>.005</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Expressed as cal/mole**
- **b** Numbers in parenthesis indicate number of animals
- **c** Probability of significant difference using unpaired Student's t-test

NS = not significant

1. Difference between control (room temperature) and aerobic, cold-acclimated groups
2. Difference between control (room temperature) and hypoxic, cold acclimated groups
Table 3. Mitochondrial Area and Shape Factor

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Normoxic, Cold-Acclimated</th>
<th>Hypoxic, Cold-Acclimated</th>
<th>$P_1^b$</th>
<th>$P_2^b$</th>
<th>$P_3^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (µm$^2$)</td>
<td>$0.236 \pm 0.012$</td>
<td>$0.506 \pm 0.023$</td>
<td>$0.656 \pm 0.040$</td>
<td>0.001</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Shape Factor</td>
<td>$0.621 \pm 0.011$</td>
<td>$0.661 \pm 0.013$</td>
<td>$0.616 \pm 0.013$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^a$ A morphometric value where 1 = to a circle and 0 = to a straight line; 150 mitochondria were measured in each group.

$^b$ Probability of significant difference using unpaired Student's t-test.

1 Difference between control (room temperature) and normoxic, cold-acclimated.

2 Difference between control (room temperature) and hypoxic, cold-acclimated.

3 Difference between normoxic, cold-acclimated and hypoxic, cold-acclimated.
Figure 1

Arrhenius plots for COX activities of control animals maintained for two weeks at room temperature. Activation energies were estimated to be 10.77 kcal mole$^{-1}$ for the temperature range of 5-35°C. Activities were higher in Lubrol-treated mitochondria, presumably due to increased exposure of COX to substrate. Correlation coefficient: .997
Figure 2

The effect of cold acclimation upon COX activities of animals allowed to surface freely (cold-normoxic). Plots of non-Lubrol treated mitochondria showed a discontinuity at 30°C, with activation energies of 10.99 kcal mole⁻¹ (5-30°C) and 5.155 kcal mole⁻¹ (30-35°C). The absence of such breaks in Lubrol-treated mitochondria suggests temperature-induced viscotropic regulation, possibly due to altered membrane phospholipids. Enzyme activities were increased approximately 20% during cold acclimation. Correlation coefficient: .996
Figure 3

The effect of hypoxic, cold acclimation upon COX activities of turtles which were forcibly submerged. "Breaks" were once again evident at 30°C in non-Lubrol treated mitochondria, with $E_a$s of 11.25 kcal mole$^{-1}$ (5-30°C) and 2.14 kcal mole$^{-1}$ (30-35°C). COX activity was approximately 25% greater than that of room temperature control animals. Correlation coefficient: .995
Cold Hypoxic

![Graph showing Log Vmax against 10^4 T (T in Kelvin) with two lines representing Lubrol and No Lubrol conditions. The graph indicates a decrease in Log Vmax as T increases.]
Figure 4a

Representative micrograph of cardiac muscle fibers of a turtle maintained at room temperature. Abundant mitochondria (M) are scattered amid glycogen reserves (G) or arranged in columns between myofibrils. A lipid droplet (L) is also apparent. Scale bar = 1 µm.
Representative micrograph of cardiac muscle fibers of a turtle maintained at room temperature. The mitochondrial population (M) is numerous and contains tightly packed cristae. Glycogen reserves (G) are nearby, scattered abundantly in the sarcoplasm. A lipid droplet (L) is apparent. Scale bar = 0.5 μm.
Figure 5a

Representative micrograph of cardiac muscle fibers of a turtle which was cold acclimated for two weeks, and allowed to surface freely. Labels are the same as for Figure 4a. Also seen within mitochondria are microcrystal inclusion bodies (C). Scale bar = 1 µm.
The volume densities of mitochondria (M) are approximately doubled area in cardiac fibers of cold-acclimated turtles, as determined by morphometric analysis. Some dense micro-crystal (C) accumulation is evident within mitochondria. Glycogen reserves (G) are present but appear to be less numerous than in warm-acclimated animals. Scale bar = 0.5 µm.
Figure 6a

Representative areas of cardiac muscle fibers from turtles acclimated to two weeks of cold-hypoxia. Labels are the same as described in Figure 4a. Z lines (Z) have been identified along with a portion of a nucleus (N). A lipid droplet (L) is apparent. Scale bar = 1 µm.
Areas of mitochondria (M) increased app. threefold in myocardia during two weeks of prolonged cold-hypoxia. While dense microcrystal inclusions (C) became more prevalent within mitochondria, glycogen granules (G) were noticeably fewer in relation to those present in control animals. Presumably these morphologic changes may be attributed to ischemia. Scale bar = 0.5 µm.
DISCUSSION

The major cardiac mitochondrial phospholipids of turtles are phosphatidylethanolamine (PE), phosphatidylcholine (PC), and diphosphatidylglycerol (DPG). These three species comprise 40.53%, 38.47%, and 14.27%, respectively, of the phosphoglyceride population in the control group (Table 1). Lesser amounts of phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (Sph) were present. The values correlate well with those for cardiac mitochondrial phospholipids of pig, sheep, cattle and humans (Tzagoloff, 1982; Daum, 1985). Of interest is the detection of a small quantity of Lysophosphatidylethanolamine (LPE), a molecule which can introduce thermodynamic disorder in the membrane.

It is fairly well documented that during cold acclimation the phospholipid population of membranes is modified in such a way that membrane fluidity and function are facilitated. This is accomplished by either decreasing fatty acyl chain lengths and/or increasing unsaturation of the fatty acids (Hazel & Prosser, 1974; Cossins, 1977; Rotermund & Veltman, 1981). While the majority of mitochondrial phospholipids remained constant in the cold, PE decreased by 8% in the cold-
acclimated (or cold-normoxic) group and 18% in the cold-hypoxic group. In contrast, DPG levels increased by 16% in the cold-normoxic group (Table 1). One could speculate that the 20% increase in COX activity in this group may be directly related to the increase in DPG. (Figure 2).

While interesting, cold induced alterations of phospholipids are not uncommon and have been reported elsewhere. For example, trout liver mitochondria show an increase in PE and a decrease in DPG (Hazel, 1979), while rat brown adipose tissue shows a decline in PC relative to PE (Senault et al., 1990). The ground squirrel erythrocyte demonstrates a decrease in both PE and PC (Rotermund & Veltman, 1981).

Generally speaking, the majority of mitochondrial phospholipids (approximately 75%) are found in the outer membrane, with DPG being the predominant phospholipid of the inner membrane (Luzikov, 1985). In contrast, the inner mitochondrial membrane contains more enzymes than phospholipids, especially the respiratory complexes for oxidative phosphorylation, ATPase, beta-hydroxybutyrate dehydrogenase, and carnitine palmityl transferase (Tzagoloff, 1982).

Cytochrome oxidase (COX) is a major component of the respiratory complex of the inner mitochondrial membrane. It is considered to be a lipid dependent enzyme requiring the presence of DPG for maximal activity (Awasthi et al., 1971;
More recently, $^{31}$P-NMR analysis has shown that lipid-rich bovine heart COX contains not only DPG (11 lipid molecules), but also 54 PE, and 64 PC molecules while the 'delipidated' complex contained 1-2 DPG's, 3-8 PE's, and 2-8 PC's (Seelig & Seelig, 1985). Calorimetric studies have shown that PE is more effective than PC at protecting COX from thermodenaturation; delipidated COX has a denaturation temperature of approximately 57° C (Yu, Gwak & Yu, 1985).

In the present investigation intact mitochondria of control, cold-normoxic, and cold-hypoxic groups exhibited comparable COX activation energies ($E_a$) of 10,770, 10,990, and 11,250 cal/mole, respectively (Table 2). The $E_a$ was decreased significantly only in the Lubrol treated cold-normoxic group, being 8,000 cal/mole ($P < 0.01$). Previously, Simon & Robin (1970) have reported that cytochrome oxidase activity declines in both heart and skeletal muscle tissue, of freshwater turtles, following a 48-hr dive at 15-18°C. Utilizing a modified method of Keilin & Hartree (1938), these investigators reported a $V_{max}$ of 4.05 moles cytochrome-c oxidized/min/mg tissue protein, in the control cardiac tissue; the apparent $V_{max}$ in the comparable group of the present investigation was approximately 9.0 moles cytochrome-c oxidized/min/mg protein. In contrast, in turtles which were allowed to acclimate at 4° C, COX activity increased significantly, by 20%, in the cold-normoxic group (Figure 2).
Figures 1, 2, and 3 graphically illustrate the increase in COX activity of the cold-normoxic plot compared with those of the control and cold-hypoxic groups. This increase of COX activity is comparable to that reported for acclimated goldfish (Tyler & Sidell, 1984).

Since torpid turtles occasionally surface during milder winter days, a quick switch from anaerobic to aerobic energy producing pathways is necessary (Gatten, 1987). This metabolic transition may illustrate the close relationship between mitochondrial lipid dependent enzymes and the phospholipid population. We can speculate that because DPG levels remain relatively constant or even increase in the cold, COX is conformationally prepared to be utilized. In fact, the increase in DPG levels parallels the increase in COX activity in the cold adapted turtles, when they can surface freely (Table 1, Fig. 2). Tzagoloff (1982) has determined that maximal activation of COX occurs at about 30% by weight of phospholipid. With the other phospholipids decreasing, the increase in DPG may be a compensatory mechanism towards this end in the cold-normoxic group.

Studies on temperature induced phase changes in mitochondrial membranes have occasionally been conflicting. Lyons and Raison (1970) have shown discontinuous Arrhenius plots for hepatic succinate oxidation in homeotherms (Sprague-Dawley albino rats), with no 'breaks', or discontinuities, in plots of poikilotherms (rainbow trout and channel catfish).
Similarly, McMurchie, Raison & Cairncross (1973), showed no breaks in Arrhenius plots of toad cardiac mitochondrial succinate oxidation, while the plots of rabbit mitochondrial oxidation did exhibit breaks. In contrast, van den Thillart & Modderkolk (1978) described discontinuities in the Arrhenius plots of state III respiration in mitochondrial preparations of goldfish liver and muscle (red and white).

The Arrhenius plots in the present investigation become curvilinear in both the cold-normoxic and cold-hypoxic groups between 25° to 30° C (Figs. 2 & 3). The cause of these 'breaks' may be due to viscotropic regulation of COX, a lipid dependent enzyme. Quite a bit of theoretical and experimental evidence suggests the discontinuities in Arrhenius plots represent thermal transitions which correspond to order-disorder transitions in the mobility of membrane lipids (Charnock, 1978). The discontinuities present in Figures 2 & 3 may be caused by a temperature induced conformation or phase change in the enzyme due to a modification of the lipid population. This is supported by the fact that there were no breaks in any of the Lubrol treated cold groups, which used COX preparations which were substantially 'delipidated'.

The significance of such alterations may be similar to that reported for a homeotherm in an investigation of succinate oxidase, H⁺ pumping and Ca²⁺ extrusion, along with succinate:cytochrome c reductase. Studies on ground squirrels have shown that the transition temperature of the Eₘ (i.e. the
temperature at which a 'break' occurs) declined from 23° C in the summer to below 3° C at a time just prior to hibernation (Aloia & Raison, 1989). This would indicate that part of the acclimation process involves mitochondrial membrane 'preparation' for the upcoming winter. In the present investigation, the linear Arrhenius plot of the control group indicates that the cardiac mitochondria of warm acclimated, or 'summer' turtles', have the capacity to function over a broad temperature spectrum. When ambient temperature declines, membrane lipids become modified to insure continued function at the lower temperatures. Although the activation energies of COX were unaltered by the cold adaptation process, the lipid changes became evident when the mitochondria of the acclimated turtles were assayed at higher temperatures; the membranes 'melt' and breaks were seen in the Arrhenius plots.

The foregoing biochemical modifications permit maintenance of cardiac function and perfusion of tissues and organs in the cold. Although cardiac output is decreased due to decreased heart rate, blood viscosity is increased due to increase in hematocrit with concomittant increases in heparin (Ultsch, 1989). During cold torpor and submergence apnea triggers a right to left shunting of blood which permits satisfactory perfusion of the brain and heart. Cholinergic stimulation causes constriction at the pulmonary outflow tract and an alteration in the cardiac depolarization center. This allows shunting to occur (Burggren, 1987).
Micrographs of cardiac tissue (Figures 5a, 5b, and 6a, 6b) illustrate that turtle heart mitochondrial area is 0.2 \( \mu m^2 \), approximately one third the area reported for those of humans (Scarpelli & Trump, 1971). During cold adaptation, glycogen reserves appear to diminish, which is consistent with the biochemical data reported previously for cold acclimated turtles (Privitera & Beall, 1969). Further, mitochondrial areas of cold acclimated turtles were increased significantly by two-fold in freely surfacing animals and by approximately three-fold during forced submergence. These size increases were accompanied by the appearance of dense intramitochondrial inclusion bodies, perhaps composed of Ca\(_3\)(PO\(_4\))\(_2\). Collectively, these changes are consistent with a state of stage 4 cellular injury due to ischemia (Scarpelli & Trump, 1971).

In turtles experiencing cold torpor, ATP becomes stabilized at lower levels (Makris & Rotermund, 1989), with anaerobic metabolism functioning to maintain quantities needed for ion pumping. The appearance of increased lactate during cold acclimation (Privitera & Beall, 1969) is attributable to an exceptionally well developed glycolytic system (Gatten, 1987). Along these lines, skeletal muscle intracellular levels of K\(^+\) decrease while levels of Na\(^+\) increase indicating leakage across cell membranes in the turtle (Ultsch, 1989). This suggests that turtle myocardia experience mild ischemia in the cold. The fact that turtle metabolism is severely depressed during cold torpor presumably makes such a
situation tolerable for extended periods at 4° C (Hochachka & Somero, 1984), but not at warm temperatures (Musacchia, 1959).

In contrast, induced ischemia, at 37° C, produced irreversible changes after 20 minutes in the cat heart (Greve, G. et al., 1990) and after 40-60 minutes in the dog heart (Jennings & Ganote, 1976). Characteristics of irreversible ischemia in these mammalian studies were mitochondrial swelling, increase in matrix space, disorganization of cristae, the appearance of amorphous matrix densities, and nuclear chromatin clumping and margination.

In contrast, those mammals which can tolerate low temperatures, such as hibernating ground squirrels, maintain membrane fluidity, permeability, and ion pumping capability with membrane receptor modification. Their hearts are resistant to fibrillation, and conduction pathways remain patent (Burlington & Milsom, 1989).

In conclusion, in the torpid turtle, frigid cold enhances changes in the mitochondrial membrane phospholipid population, which may affect intricately associated enzymes such as COX, whose activity increases. These changes, along with a well developed extramitochondrial system of anaerobic glycolysis, produce the energy needed to sustain cardiac function at diminished levels during periods of lower oxygen tension. Although reversible ischemia occurs, it is at tolerable levels for survival of the species in the cold.
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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

12-9-91

Date

Director’s Signature