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AN ABSTRACT OF THE THESIS OF Mark Lowell Wagner for the
Master of Science in Biology presented March 27, 1989.

Title: The Physiology and Biochemistry of Isolated Skeletal Muscle
Mitochondria: A Comparative Study.

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE:

Malcolm S. Lea, Chair

Stanley S. Hillman

Richard B. Forbes

Alfred S. Levinson

The physiological limit to maximum aerobic capacity ($\dot{V}O_{2\max}$) in

vertebrates has been attributed to cardiovascular oxygen delivery, to the ability of the muscle cells to consume oxygen, or to a fine-tuned development of all components of the respiratory system such that no single component can be shown to limit $\dot{V}O_2\text{max}$. The above hypotheses have each been developed using different experiments with different animals. The comparative studies uniting these animals and methods are limited. In order to further our knowledge of the cellular limit to $\dot{V}O_2\text{max}$, skeletal muscle mitochondria were isolated from species representing four vertebrate classes, and endothermic and ectothermic physiology. Mitochondrial $\dot{V}O_2$ was measured at 15, 25 and 35°C and the results were compared between species and endothermic and ectothermic groups. Mitochondrial enzyme activities were measured at the three treatment temperatures to ascertain which enzyme activity best represents $\dot{V}O_2\text{max}$ for all vertebrates. Cytochrome difference spectra were measured to determine the concentrations of mitochondrial cytochromes c+c₁. The results show that mitochondria are unique in all species tested. Each species has its own response to changing temperature and its own mitochondrial enzyme activity profile. In addition, *in vitro* measurements of mitochondrial $\dot{V}O_2$ for all species show rates significantly higher than those estimated from whole organism measurements of

$\dot{V}O_{2\max}$, suggesting that mitochondrial oxygen uptake is not a factor limiting organismal $\dot{V}O_{2\max}$. The Q_{10} for mitochondrial $\dot{V}O_2$ differed significantly between groups, indicating that differences in $\dot{V}O_{2\max}$ between endotherms and ectotherms cannot be explained solely on the basis of temperature. The activation energy (E_a) of mitochondrial $\dot{V}O_2$ was significantly higher in endotherms compared to ectotherms. Mitochondrial enzyme activities did not show the same Q_{10} and E_a differences as the intact mitochondria. Since enzyme activities were measured on mitochondria disrupted with either detergent or sonication, physical properties of the mitochondrial inner membrane are suggested as being responsible for these differences.

THE PHYSIOLOGY AND BIOCHEMISTRY OF ISOLATED
SKELETAL MUSCLE MITOCHONDRIA:
A COMPARATIVE STUDY

by

MARK LOWELL WAGNER

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

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1989

TO THE OFFICE OF GRADUATE STUDIES:

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TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
CHAPTER	
I INTRODUCTION	1
II MATERIALS AND METHODS	9
Materials	9
Methods	9
III RESULTS	16
IV DISCUSSION	46
V CONCLUSIONS	54
VI LITERATURE CITED	56
VII APPENDIX	64

LIST OF TABLES

TABLE		PAGE
I	Summary of Mitochondrial $\dot{V}O_2$	18
IIa	Summary of Mitochondrial Enzyme Activities per Gram Wet Weight Muscle	25
IIb	Summary of Mitochondrial Enzyme Activities per nmol Cytochrome c+c ₁	26
III	Activation Energies of Mitochondrial Enzymes	39
IV	Mitochondrial Enzyme and $\dot{V}O_2$ Activity Ratios	40

LIST OF FIGURES

FIGURE	PAGE
1a. Mitochondrial $\dot{V}O_2$ per gram wet weight muscle	19
1b. Mitochondrial $\dot{V}O_2$ per nmol cytochrome c+c ₁	20
2. Log/log plot of mitochondrial $\dot{V}O_2$ vs. temperature	21
3. Arrhenius plot of mitochondrial $\dot{V}O_2$	22
4. Cytochrome c oxidase activity per gram wet weight muscle	27
5. Succinate cytochrome c reductase activity per gram wet weight muscle	28
6. Succinate dehydrogenase activity per gram wet weight muscle	29
7. NADH oxidase activity per gram wet weight muscle	30
8. Citrate synthase activity per gram wet weight muscle	31
9. Cytochrome c oxidase activity per nmol cytochrome c+c ₁	32
10. Succinate cytochrome c reductase activity per nmol cytochrome c+c ₁	35
11. Succinate dehydrogenase activity per nmol cytochrome c+c ₁	36

12. NADH oxidase activity per nmol cytochrome c+c ₁	37
13. Citrate synthase activity per nmol cytochrome c+c ₁	38
14. Mitochondrial $\dot{V}O_2$ vs COX activity	41
15. Mitochondrial $\dot{V}O_2$ vs SCc activity	42
16. Mitochondrial $\dot{V}O_2$ vs SDH activity	43
17. Mitochondrial $\dot{V}O_2$ vs NADHOX activity	44
18. Mitochondrial $\dot{V}O_2$ vs CS activity	45

INTRODUCTION

Most of the adenosine triphosphate (ATP) required by vertebrates for locomotion is generated by skeletal muscle mitochondria. Mitochondria require oxygen to generate and maintain the chemiosmotic gradient that drives ATP synthesis. During maximal aerobic activity skeletal muscle mitochondria utilize more than 90% of the oxygen consumed by an organism (Weibel, 1979). An organism's maximum aerobic capacity ($\dot{V}O_{2\max}$) can be measured accurately by determining the amount of oxygen consumed during activity. Maximum aerobic capacity can be estimated by measuring the $\dot{V}O_2$ of muscle homogenates or mitochondria isolated from skeletal muscle, or it can be extrapolated from $V_{\max}$ of mitochondrial enzymes.

The physiological limit to $\dot{V}O_{2\max}$ is highly controversial. Saltin (1985) has summarized three distinct views on the limit to maximal aerobic capacity: 1) the limit is a function of cardiovascular ability to deliver oxygen to muscle cells (Hillman 1976, 1980, 1982; Hillman, et. al., 1985; Saltin, 1985; Walsberg, et. al., 1986; Schultz, 1988); 2) the limit is within the cells; i.e., the ability of the mitochondria to utilize available oxygen (Holloszy and Booth, 1976; Hoppeler, et.al. 1981a,b; Mathieu, et. al., 1981;

Hoppeler and Lindstedt 1985; Hoppeler, et. al., 1987) and 3) the limit to $\dot{V}O_2\text{max}$ cannot be attributed to any one single factor because the components of the system are so closely interconnected or the means of measurement are too imprecise to produce a clear answer (Weibel, et. al., 1981; Di Prampero, 1985; Taylor, et. al., 1987a,b).

Saltin (1985) measured $\dot{V}O_2\text{max}$ in isolated quadriceps muscle of humans. He concluded, on the basis of tissue blood flow at maximal activity, that to perfuse equally the entire muscular system would require a cardiac output of 50 to 60 liters per minute. This is approximately twice the average maximal cardiac output of the human heart (Blomqvist and Saltin, 1983). Walsberg, et. al. (1986) measured $\dot{V}O_2\text{max}$ in wild bullfrogs (*Rana catesbeiana*) and found a two-fold variation closely correlated to ventricular mass and hemoglobin concentration. This variation did not correlate with citrate synthase activity, a proposed indicator of aerobic capacity (Holloszy and Booth, 1976; Putnam and Bennett, 1983), suggesting that cardiovascular oxygen delivery is the limit to $\dot{V}O_2\text{max}$ in anuran amphibians. This finding supports the work of Hillman (1976, 1980, 1982; Hillman, et al, 1985), who experimentally varied cardiac output and hemoglobin concentration in several species of anurans and found them closely correlated with changes in $\dot{V}O_2\text{max}$. Hillman's work has been criticized as not accurately measuring

$\dot{V}O_2\text{max}$ because of limitations of his experimental method for eliciting maximal aerobic activity (Marsh and Taigen, 1987). Data extrapolated from calling activity in male gray tree frogs (*Hyla versicolor*) indicate that $\dot{V}O_2\text{max}$ during calling is higher than during forced activity (Marsh and Taigen, 1987). Marsh and Taigen (1987) may have measured a species-specific effect, however, since Hillman, et. al. (1985) found $\dot{V}O_2\text{max}$ to be proportional to oxygen transport capacity independent of sex in the toad, *Bufo marinus*.

It has been shown in mammals of very different body mass that the estimated total number of mitochondria in an organism can be used as a predictor of $\dot{V}O_2\text{max}$ (Hoppeler, et. al., 1981a,b; Mathieu, et. al.,1981; Hoppeler and Lindstedt, 1985; Hoppeler, et. al.,1987; Hudlicka, et. al., 1988). Taylor, et. al. (1981) reported that for several African mammals ranging in mass from 0.5 to 250 kilograms, $\dot{V}O_2\text{max}$ varies as the 0.75 power of mass. While there is great diversity in the distribution of mitochondria per muscle and per species, the total mitochondrial number correlates with mass-specific $\dot{V}O_2\text{max}$ (Mathieu, et. al., 1981). Based on data from several mammalian species, including humans and one bird, Hoppeler and Lindstedt (1985) calculated the mitochondrial $\dot{V}O_2\text{max}$ to be 3-5ml $O_2/\text{min}\cdot\text{ml}$ mitochondria. They state that organismal $\dot{V}O_2\text{max}$ can be estimated from the total number of mitochondria in a muscle

sample extrapolated to total muscle mass and mitochondrial volume. This finding implies that mitochondria respire at the same rate regardless of body mass or species, at least for those species examined by Hoppeler and Lindstedt (1985). In contrast, recent data suggest that while mitochondrial volume density (mitochondrial volume per muscle fiber volume) and capillary length density (capillary length per muscle fiber volume) are linearly related, they may not be the only factors responsible for differences in $\dot{V}O_2\text{max}$. For a 2.5-fold difference in aerobic capacity between aerobic ponies and dogs, and less aerobic calves and goats, only 1.7 times more capillarity was found; the balance of the difference was a 1.5 times higher hematocrit in the aerobic mammals (Hoppeler and Kayar, 1988).

Di Prampero (1985) and Weibel, et. al., (1981) contend that the respiratory system in mammals is so tightly integrated that to isolate one particular component as the definitive limiting factor for $\dot{V}O_2\text{max}$ is very difficult. Considerable plasticity is inherent in the respiratory system. The individual components respond differently, but in a coordinated manner - a phenomenon termed symmorphosis- to meet the aerobic demands of the organism (Taylor and Weibel, 1981; Taylor, et. al., 1987a,b). Di Prampero's (1985) conclusion is that the limit to whole body $\dot{V}O_2\text{max}$ depends

upon cardiac output, however, the way maximal activity is measured may influence the apparent cardiovascular involvement. During one-legged exercise training in man, where relatively few muscle groups are involved, changes in $\dot{V}O_{2\max}$ were dependent in approximately equal parts on changes in cardiac output and perfusion of the muscle by the peripheral circulation. Changes in mitochondrial oxidative capacity, measured on the basis of succinate dehydrogenase activity, were in excess of the overall change in $\dot{V}O_{2\max}$. Changes in whole body $\dot{V}O_{2\max}$, he concludes, were limited mostly by cardiac output (about 80%), while tissue perfusion and mitochondrial oxidative capacity had only a minor involvement in the overall limit to $\dot{V}O_{2\max}$.

One weakness in the arguments presented above is that the bulk of the evidence supporting the cellular limit and symmorphosis theories is based on data from experiments on mammals, the evidence for a cardiovascular limit to aerobic capacity is derived mainly from experiments on amphibians. Considering the physiological differences between these groups, a simple model may not suffice to describe limits to aerobic capacity in all animals. This study compares the physiological and biochemical properties of skeletal muscle mitochondria from endotherms and ectotherms. Such information is essential to any comparative study of aerobic

physiology and may shed light on the limiting factor in aerobic capacity.

Previous studies have shown that the temperature-dependent responses of mitochondrial oxidation from vertebrates of different classes vary as a function of acclimation temperature and class (Lyons and Raison, 1970; Raison, et. al., 1971a,b; Hazel 1972a,b; Roberts et. al., 1972; Pye, 1973; Smith, 1973a,b,c; Pye, et. al., 1976; McMurchie and Raison, 1979; Ohira and Ohira, 1988; Raison, et. al., 1988; Wodtke, 1973, 1974a,b, 1976, 1981). A wide variety of vertebrate mitochondria from liver, heart and skeletal muscle show a discontinuous Arrhenius plot of enzyme activation energy. This discontinuity has been attributed to a temperature induced phase transition in the phospholipid portion of the mitochondrial inner membrane. Roberts, et. al., (1972) found a higher ADP/O ratio and lower Q_{10} in mitochondria from hibernating mammals compared with non-hibernating mammals, suggesting an adaptation for maintaining metabolic function during hibernation and rewarming. Raison, et.al. (1988) found seasonal changes in the transition temperature (T^*) of membrane phospholipids in liver mitochondria from the golden-mantled ground squirrel (*Spermophilus lateralis*). In contrast, cardiac muscle mitochondria from the same animal did not show this seasonal change: T^* was maintained at a temperature well

below the minimum body temperature of the hibernating animal ($<4^{\circ}\text{C}$). They (Raison, et.al., 1988) propose that heterothermic endotherms have adaptations which are tissue-specific. Wodtke (1974a,b, 1976, 1981) found species- and tissue-specific differences in succinate, β -hydroxy butyrate, and glutamate oxidation in fish. Cold acclimation decreased $\dot{V}\text{O}_{2\text{max}}$ in liver mitochondria but increased $\dot{V}\text{O}_{2\text{max}}$ in skeletal muscle mitochondria. Pye, et. al. (1976) reported that cold acclimation in a fish (Tench, *Tinca tinca*) leads to a shift in *in vitro* substrate utilization dependent upon the tissue studied. For example, liver mitochondria respire maximally in the presence of succinate, while skeletal muscle mitochondria maintain a maximum rate of respiration using α -ketoglutarate as substrate. Ohira and Ohira (1988) found increased mitochondrial enzyme activities and decreased ATP concentrations, but no effect on the rate of glycolysis in 4°C acclimated frogs. The data suggest substantial differences in the temperature-dependent properties of mitochondria from various vertebrate classes and between species within the same class. These findings indicate that great care must be taken when interpreting $\dot{V}\text{O}_{2\text{max}}$ in different classes of vertebrates.

While there has been an abundance of work comparing the aerobic capacity of vertebrates within a particular class, very little

work has involved comparisons between classes of vertebrates or between ectotherms and endotherms in general (Alp, et. al., 1976; Driedzic, et.al.,1987). It is essential to any comparative statement on aerobic metabolism that the criteria used to judge variability, limitations and capacity be based on indices which represent $\dot{V}O_2\text{max}$ independent of class. Since mitochondria are the terminal oxygen acceptors, they can be used to estimate the aerobic potential of the tissue from which they were isolated. Therefore, the objectives of this study are:

- 1) to describe the physiological and biochemical responses of skeletal muscle mitochondria from four vertebrate classes to temperature change,
- 2) to present an enzyme activity profile of the muscle mitochondria and
- 3) propose an enzyme assay that best represents the physiological $\dot{V}O_2\text{max}$ of the muscle mitochondrion independent of class.

MATERIALS AND METHODS

MATERIALS

All inorganic salts and acids used were of reagent grade and obtained from various sources. Organic compounds were of the highest available purity and obtained from Sigma Chemical Company. Animals were obtained from either local sources (rats, *Rattus norvegicus* and rock doves, *Columba livia*); provided by Dr. S. S. Hillman (giant toads, *Bufo marinus*), or caught at a local trout farm (rainbow trout, *Salmo gairdneri*) All animals were adults.

METHODS

Mitochondrial isolation:

Mitochondria were prepared from skeletal muscle involved in locomotion (thigh muscles from rats and toads, breast muscle from the birds, epaxial muscle from the trout). To ensure adequate mitochondria for study, muscle from toads and fish was pooled from two individuals for each mitochondrial preparation. All animals were killed either by decapitation after mild CO₂ anesthesia (rats and birds) or by pithing (toads and fish). Skeletal muscle was quickly removed, placed in a preweighed beaker of ice cold sucrose wash

medium (SET medium: 250mM Sucrose, 10mM Tris-HCl , 2mM EDTA: pH 7.4) and the wet weight determined. The tissue was coarsely chopped with scissors and the medium poured off. The tissue was rinsed once with SET medium, resuspended in one volume (1ml per gram wet weight tissue) SETH medium (SET with 50IU/ml sodium heparin) and chopped finely with scissors. The volume was doubled with SETH, then trypsin (Sigma type: III, 0.5mg/gram wet tissue weight) was added and the suspension stirred gently in an ice-water bath for 30 minutes, after which Soybean Trypsin Inhibitor (Sigma type: I-S, 0.5mg/g wet wt.) was added and the suspension diluted to twenty volumes. The tissue digest was then homogenized by 3 bursts of ten seconds each with a mechanical tissue homogenizer (Polytron). Mitochondria were then isolated by the modified Bookleman method described by Fischer, et. al. (1985a). The homogenate was centrifuged at 600g for 10min and the resultant supernatant centrifuged at 10,000g for 10min to yield a mitochondrial pellet. This crude mitochondrial pellet was resuspended in 20ml SETH and centrifuged for 10min at 1000g. The resultant supernatant was centrifuged 10min at 10,000g. The pellet was resuspended in 10ml SET and centrifuged again at 10,000g for 10min. The final pellet was diluted with SET (0.05ml/g wet wt tissue) and gently homogenized with a ground glass pestle to disperse the pellet. Aliquots taken for biochemical analysis were

frozen immediately on dry ice; the remaining sample was placed in an ice bath until used.

Polarography:

The oxygen uptake medium consisted of 0.15M KCl, 0.01M Tris-HCl and 0.03M KPi at pH 7.4 as measured at room temperature. Oxygen uptake studies were conducted at 15, 25 and 35°C in a water-jacketed cell equipped with a Clark-type oxygen electrode and a Yellow Springs oxygen monitor (model 55). A 50 μ l aliquot of mitochondria was added to 1.3ml of air-equilibrated oxygen uptake medium and the chamber was sealed. Substrate (glutamate/ malate) and ADP were added through a 1mm diam. capillary opening. The final substrate concentrations were 10mM glutamate and 5mM malate. The final concentration of ADP was 370 μ M. The concentration of oxygen in solution was determined at 15°, 25° and 35°C by NADH oxidation using sonicated beef heart mitochondria resuspended in oxygen uptake medium as described by Chappell (1964).

Enzyme Assays:

All enzyme assays were conducted in a Beckman DU7 UV/Vis spectrophotometer equipped with a temperature controlled cuvette holder. Unless otherwise specified, assays were conducted at 35, 25 and 15°C on once-thawed mitochondria diluted to 1mg protein/ml

with SET containing 0.1% Tween 80 and kept on ice until used.

Cytochrome c oxidase activity was assayed by a modified version of the the method of Wharton and Tzagoloff (1967) as described by Darley-Usmar, et. al.(1983). Samples were allowed to incubate in the reaction medium (50mM Phosphate buffer with 0.5% Tween 80, pH 7.0) for 5 min at 30°C before beginning the reaction by the addition of ferrocytochrome c. The initial concentration of ferrocytochrome c was 10 μ M .

Activities of succinate dehydrogenase and succinate: cytochrome c reductase were estimated as described by Fischer et. al. (1985b). The mitochondria were activated before assay by incubation for 30min at 30°C in Phosphate/Tween buffer (50mM potassium phosphate/0.5% Tween-80, pH 7.4) with 20mM succinate after which the suspension was stored on ice and aliquots taken for assay as described below. The final concentration of Tween in the assay was 0.01%.

Succinate: cytochrome c reductase was assayed by following the reduction of ferricytochrome c at 550nm. A millimolar extinction coefficient of 27.6 was used (Fischer, et. al., 1985b).

Succinate dehydrogenase activity was estimated by following the reduction of 2,6-dichlorophenol indophenol (DCIP) at 600nm. Phenazine methosulfate (1mM) was used as the primary electron acceptor. The millimolar extinction coefficient for reduced DCIP is

19.1 (Fischer, et. al., 1985b).

For NADH oxidase mitochondria were diluted to 1mg/ml in SET and the rotenone sensitive (electron transport) activity determined by the reduction of NADH at 340nm as described by Fischer, et. al. (1986). The millimolar extinction coefficient for NADH is 6.22 (Fischer, et. al., 1986).

Citrate synthase activity was estimated according to a modified method from Srere (1969) as described by Putnam and Bennett (1983). Mitochondria diluted to 1mg/ml with SET with 0.1% Tween 80 were frozen/thawed 3 times before use. The assay follows the reduction of dithio-*bis*-nitrobenzoic acid (DTNB) at 412nm. The millimolar extinction coefficient for reduced DTNB is 13.6 (Srere, 1969).

Cytochrome c+c₁ concentrations were determined on mitochondria diluted to 1mg/ml with Phosphate/Tween buffer (50mM phosphate/ 0.5% Tween-80, pH 7.0). Difference spectra (oxidized minus reduced) were conducted at 30°C. Samples were reduced by addition of 10mM Succinate (pH 7.4) and a few grains of sodium dithionite. Cytochrome c+c₁ concentrations were calculated as described by Bookelman, et. al. (1978).

Mitochondrial yield was determined by comparing the citrate synthase activity of the muscle homogenate and of the isolated mitochondria at 25°C as described above. Muscle homogenate was

diluted 1:10 with SET/Tween-80 and prepared as described above.

Protein concentrations were determined by the method of Lowry, et. al. (1951) with defatted crystalline BSA used as a standard. The final reaction mixture for protein determination contained 0.8% sodium dodecyl sulfate (SDS). Protein concentrations were calculated according to the method of Cookley and James as described in Peterson (1977).

The data are expressed in units ($\mu\text{moles/min}$) per nmole cytochrome c+c₁ or per gram wet weight muscle as calculated from yield determinations which are based on assays of citrate synthase activity in the muscle homogenate and crude mitochondrial pellet. Yield determinations could not be based on homogenate cytochrome concentration due to interference from non-mitochondrial cytochromes which absorb light at or near the absorbance range of mitochondrial cytochrome c+c₁. For comparison with published figures for organismal VO_2max , mitochondrial VO_2 is also used to calculate ml O₂ per hour per gram body weight as described below (see Discussion). A respiratory control ratio (RCR) was determined immediately after mitochondrial isolation by the method of Chance and Williams (1959), except that to ensure maximum oxygen uptake rates for mitochondria from all animals studied, ADP and substrate were added in such excess that State IV (high [substrate], low [ADP], high [ATP]) respiration was not attained; therefore control ratios

are rates of oxygen uptake of State III (high [substrate], high [ADP]) divided by State II (high [substrate], no ADP) as described by Anderson (1986). Preparations with RCRs below six were discarded.

Activation energies were calculated from Arrhenius plots of mitochondrial $\dot{V}O_2$ and enzyme activity as described in Lyons, et. al. (1974).

Statistics:

Data were tested for statistical difference with Student's two-tailed t-test and one- and two-way ANOVA using the StatWorks™ statistical package on an Apple Macintosh. Differences were considered statistically significant at or above the 95% confidence interval.

RESULTS

Mitochondrial $\dot{V}O_2$, measured at the three treatment temperatures and expressed as described above, is summarized in Table I. Mitochondrial $\dot{V}O_2$ per gram muscle (Figure 1a) differs significantly ($p \leq 0.05$) between the endotherms and ectotherms tested at all treatment temperatures. However, when expressed per nmole cytochrome c+c₁ (Figure 1b) there are significantly greater differences ($p \leq 0.05$) in $\dot{V}O_2$ between the rock doves and rats, and between the trout and toads, than between endotherms and ectotherms. Mitochondrial $\dot{V}O_2$ in rock doves was significantly lower ($p \leq 0.001$) than in all other animals tested.

Double logarithmic plots of $\dot{V}O_2$ and temperature (Figure 2) show significantly steeper slopes ($p \leq 0.01$) for endotherms compared to ectotherms; in addition the trout had a significantly lower slope than the toads ($p \leq 0.05$).

The endotherms had significantly higher Q_{10} values in each temperature range than ectotherms ($p \leq 0.01$; Table 1). Over the temperature range studied, Arrhenius plots of mitochondrial $\dot{V}O_2$ show significantly higher ($p \leq 0.001$) activation energies (E_a) in endotherms than in ectotherms (Figure 3, Table I). There are no significant differences in E_a or Q_{10} between the species within

either endothermic or ectothermic groups.

TABLE I

SUMMARY OF MITOCHONDRIAL $\dot{V}O_2$

Animal	Temp (°C)	nmol O ₂ /min /nmol oc1	nmol O ₂ /min /gram muscle	ml O ₂ /hour /gram muscle	ml O ₂ /hour /gram body wt (e)	ml O ₂ /hour /gram body wt (f)	Ea	Q10
<i>Rattus norvegicus</i> (n=5)	35	582 (204)	2194 (554)	32.2 (8.1)	14.3 (3.6)	4.6 (0.1) a	21.1 (0.3)	2.4(0.2)
	25	229 (52)	907 (369)	13.3 (5.4)	5.9 (2.4)	(<i>Rattus norvegicus</i>)		4.9(0.5)
	15	53 (17)	206 (59)	3.0 (0.9)	1.3 (0.4)			
<i>Columba livia</i> (n=5)	35	100 (36)	2621 (709)	38 (10)	16.9 (4.4)	11.9 (Tb=43°C) b	21.0 (1.8)	2.(0.2)
	25	42 (16)	1180 (287)	17 (4)	7.6 (1.8)	(<i>Columba livia</i>)		5.0(0.5)
	15	10 (3)	282 (71)	4 (1)	1.8 (0.4)			
<i>Bufo marinus</i> (n=4)	35	131 (33)	444 (256)	7 (4)	3.1 (1.8)	0.7 (30°C) c	12.2 (3.0)	1.9(0.2)
	25	69 (8)	204 (72)	3 (1)	1.3 (0.4)	0.3 (20°C) c		2.5(0.3)
	15	30 (9)	84 (25)	1.3 (0.4)	0.6 (0.2)	(<i>Bufo marinus</i>)		
<i>Salmo gairdneri</i> (n=4)	35	259 (52)	296 (47)	4 (1)	1.8 (0.4)		9.5 (0.4)	1.3(0.1)
	25	211 (46)	234 (16)	3 (0.2)	1.3 (0.1)	0.7 (20°C) d		2.4(0.4)
	15	88 (13)	101 (14)	2 (0.2)	0.9 (0.1)	(<i>Onchorhynchus sp</i>)		

Values are means $\pm$ standard error, sample sizes are also given. a. Davies, et. al. (1981); b. Butler, et. al. (1977);

c. Hedrick (1985); d. Brett and Groves (1979); e. values calculated from mitochondrial $\dot{V}O_2$ data;

f. values of whole animal $\dot{V}O_{2max}$ from the literature.

Figure 1a. Mitochondrial VO_2 per gram wet weight muscle. Values are means $\pm$ standard error and are based on measurements of mitochondrial VO_2 and yield determinations (see text for discussion of method).

Figure 1b. Mitochondrial $\dot{V}O_2$ per nmol cytochrome c+c1. Values are means $\pm$ standard error and are based on measurements of mitochondrial $\dot{V}O_2$ and cytochrome concentration (see text for discussion of method).

Figure 2. Log/log plot of mitochondrial $\dot{V}O_2$ versus temperature.

Figure 3. Arrhenius plot of mitochondrial VO_2 .

Table II summarizes mitochondrial enzyme activities. The enzyme activities represent components of the electron transport system (ETS) and tricarboxylic acid (TCA) cycle. Citrate synthase is an enzyme of the TCA cycle located in the mitochondrial matrix. Cytochrome c oxidase is Complex IV of the ETS, succinate: cytochrome c reductase comprises complexes II and III of the ETS while succinate dehydrogenase consists of two subunits (SD₁ and SD₂) of complex II. NADH oxidase measures the activity of complexes I, III and IV of the ETS as a complete system. All ETS enzymes are located in or on the mitochondrial inner membrane. For all enzymes tested and at all temperatures, activities per gram were significantly higher in birds ($p \leq 0.001$) than in all other animals (Table IIa and Figures 4-8).

Where possible, enzyme activities were compared to published values. The activities of CS, SDH and COX were comparable to published figures (Alp, et. al., 1979; Davies, et. al., 1981; Putnam and Bennett, 1983). NADHox activity measured in rat muscle mitochondria was an order of magnitude higher than reported by Davies, et al. (1981). Their activity, however, represents a measurement from intact mitochondria while the activity reported here is determined on a sonicated mitochondrial preparation allowing increased availability of NADH to the oxidase system.

Activities expressed per nmole cytochrome c+c₁ (Table IIb)

showed a more variable pattern. Rainbow trout have significantly higher cytochrome c oxidase (COX) activity per nmol cytochrome c+c₁ than all other animals at all temperatures (Figure 9).

TABLE IIa

SUMMARY OF MITOCHONDRIAL ENZYME ACTIVITIES

Animal	Temp (°C)	COX	S:Cc	SDH	NADHOX	CS
Mammal	35	49.0 (6.1)	6.6 (0.8)	9.4 (1.0)	5.6 (0.7)	14.5 (0.7)
	25	29.5 (2.3)	4.3 (0.5)	5.1 (0.5)	2.7 (0.4)	10.8 (0.3)
	15	18.7 (2.3)	2.8 (0.3)	2.7 (0.3)	1.4 (0.3)	5.3 (0.3)
Bird	35	386.2 (22.4)	53.7 (2.5)	69.8 (4.2)	85.9 (4.7)	165.1 (6.6)
	25	216.3 (14.3)	33.2 (1.8)	37.6 (2.3)	45.1 (2.6)	119.1 (4.3)
	15	146.0 (12.5)	23.1 (1.2)	21.4 (1.0)	21.2 (1.7)	77.0 (3.3)
Toad	35	31.6 (4.1)	1.6 (0.2)	5.2 (0.5)	3.5 (0.5)	28.6 (2.8)
	25	20.6 (3.0)	1.1 (0.1)	3.3 (0.3)	1.8 (0.4)	20.3 (1.5)
	15	12.0 (1.7)	0.7 (0.2)	2.1 (0.3)	0.8 (0.1)	10.9 (0.7)
Trout	35	21.7 (3.4)		1.2 (0.3)	0.8 (0.1)	6.7 (0.8)
	25	16.0 (1.7)		0.6 (0.1)	0.5 (0.1)	5.9 (1.0)
	15	9.3 (1.2)		0.5 (0.1)	0.4 (0.1)	4.4 (0.8)

(Values are means with standard errors in parentheses ($\mu\text{mol/min gram wet weight muscle}$).

TABLE IIb

SUMMARY OF MITOCHONDRIAL ENZYME ACTIVITIES

Animal	Temp (°C)	COX	S:Cc	SDH	NADHOX	CS
Mammal	35	12.1(1.2)	1.9 (0.4)	2.5 (0.3)	1.5 (0.2)	4.3 (0.8)
	25	7.3 (0.7)	1.0 (0.2)	1.3 (0.2)	0.7 (0.1)	3.2 (0.7)
	15	4.6(0.5)	0.8 (0.1)	0.7 (0.1)	0.3 (0.1)	1.6 (0.3)
Bird	35	14.0 (0.8)	2.0 (0.1)	2.5 (0.1)	3.0 (0.1)	6.1(0.5)
	25	7.8 (0.4)	1.2 (0.1)	1.3 (0.1)	1.5 (0.1)	4.5 (0.3)
	15	5.2 (0.2)	0.8 (0.1)	0.8 (0.1)	0.7 (0.1)	2.9 (0.2)
Toad	35	10.4 (0.6)	0.6 (0.1)	1.9 (0.3)	1.2 (0.1)	10.2 (1.2)
	25	6.7 (0.3)	0.4 (0.1)	1.2 (0.2)	0.6 (0.1)	7.3 (0.8)
	15	4.0 (0.4)	0.2 (0.1)	0.8 (0.1)	0.3 (0.1)	4.1 (0.5)
Trout	35	18.3 (1.4)		1.0 (0.1)	0.7 (0.1)	5.8 (0.4)
	25	13.7 (0.5)		0.5 (0.1)	0.5 (0.1)	5.0 (0.3)
	15	7.9 (0.3)		0.4 (0.1)	0.4 (0.2)	3.7 (0.2)

(Values are means with standard errors in parentheses ($\mu\text{mol/min nmol cytochrome c+c1}$)).

Figure 4. Cytochrome c oxidase activity expressed per gram wet weight muscle. Values are means $\pm$ standard error.

Figure 5. Succinate cytochrome c reductase activity expressed per gram wet weight muscle. Values are means $\pm$ standard error.

Figure 6. Succinate dehydrogenase activity expressed per gram wet weight muscle. Values are means $\pm$ standard error.

Figure 7. NADH oxidase activity expressed per gram wet weight muscle. Values are means $\pm$ standard error.

Figure 8. Citrate synthase activity expressed per gram wet weight muscle. Values are means $\pm$ standard error.

Figure 9. Cytochrome c oxidase activity expressed per nmol cytochrome c+c1. Values are means $\pm$ standard error.

The endotherms have significantly higher succinate: cytochrome c reductase (SCc) activities than do the ectotherms (Figure 10); SCc activity was too low to distinguish from background scattering in trout mitochondria. Succinate dehydrogenase (SDH) activity is significantly lower ($p \leq 0.001$) in trout, compared to all other animals tested and lower in the ectotherms than in the endotherms at 35°C (Figure 11). NADH oxidase (NADHOX) activity was highest in the birds at 35° and 25°C (figure 12) and similar in all species tested at 15°C. Citrate synthase (CS) activity per nmol c+c₁ is significantly higher in toads at 35° and 25°C (Figure 13) and similar in all species tested at 15°C. Values for Q₁₀ were not significantly different among the enzymes studied.

Enzyme activation energies (E_a) for the endotherms and ectotherms were not significantly different for COX, CS or NADHOX (Table III). The E_a for SDH was significantly higher ($p \leq 0.001$) in the endotherms than the ectotherms. The E_a for CS was significantly ($p \leq 0.05$) lower in trout than in toads and lower in rock doves than in rats ($p \leq 0.05$), while rats and toads had similar activation energies. The slopes of double logarithmic plots of enzyme activity versus temperature (not shown) were not significantly different for COX, SCc and SDH. Trout had significantly lower ($p \leq 0.05$) slopes for NADHOX and CS when compared to the other animals.

Activity ratios were calculated to establish patterns which

might not be apparent from analysis of comparative data (Table IV). The ratios of NADHOX to CS, SDH to CS, CS to COX and NADHOX to COX were significantly higher in endotherms compared to ectotherms ($p \leq 0.05$). While the ratio of SDH activity to COX activity differed significantly in endotherms compared to ectotherms; this difference was due to a significantly lower ratio in the trout when compared to all other animals tested, while the toads were not significantly different from the endotherms. Other ratios differed between species and are summarized in Table IV.

The relationship between enzyme activity and $\dot{V}O_2$ was examined in order to determine an enzyme assay that best represents mitochondrial $\dot{V}O_2$, for all animals studied. Least squares regression analyses of $\dot{V}O_2$ versus enzyme activity for all groups are presented in Figures 14 through 18. For $\dot{V}O_2$ versus COX and CS, the mammal differed significantly ($p \leq 0.05$) from all other groups; the trout had significantly higher ($p \leq 0.05$) slopes with respect to CS activity than the birds or toads. The birds had significantly lower ($p \leq 0.05$) slopes than all other animals and the toads were lower than rats and trout for $\dot{V}O_2$ versus SCc, SDH and NADHOX. Activities for $\dot{V}O_2$ versus SDH and NADHOX from mammals and fish were not significantly different.

Figure 10. Succinate cytochrome c reductase activity expressed per nmol cytochrome c+c₁. Values are means $\pm$ standard error.

Figure 11. Succinate dehydrogenase activity expressed per nmol cytochrome c+c1. Values are means $\pm$ standard error.

Figure 12. NADH oxidase activity expressed per nmol cytochrome c+c1. Values are means $\pm$ standard error.

Figure 13. Citrate synthase activity expressed per nmol cytochrome c+c₁. Values are means $\pm$ standard error.

TABLE III
ACTIVATION ENERGIES OF MITOCHONDRIAL ENZYMES
(KCAL)

Animal	COX	S:Cc	SDH	NADHox	CS
Mammal	8.5 (0.1)	7.9 (0.5)	11.3 (0.6)**	13.7 (2.6)	9.0 (0.7)
Bird	9.0 (0.9)	7.6 (0.4)	9.8 (0.5)**	13.1 (0.8)	6.9 (0.2)
Toad	8.6 (0.5)	8.0 (0.6)	8.0 (0.6)	12.0 (1.4)	8.4 (0.6)
Trout	7.4 (0.3)*		7.8 (0.1)	6.8 (4.6)*	4.0 (1.3)*

Values are means with standard errors in parentheses

* significantly different from all other animals ($p < 0.05$)

** endotherms significantly different from ectotherms ($p < 0.05$)

TABLE IV

MITOCHONDRIAL ENZYME AND VO₂ ACTIVITY RATIOS

Ratio	Temp	Mammal	Bird	Toad	Fish
VO ₂ /COX	35	4.6	0.7	1.3	1.4
	25	3.1	0.6	1.0	1.5
	15	1.1	0.9	0.7	1.1
VO ₂ /SDH	35	21.4	3.9	7.8	25.4
	25	17.4	3.1	6.0	39.1
	15	7.8	1.3	3.9	21.1
VO ₂ /CS	35	14.4	1.6	1.4	4.5
	25	7.7	1.0	1.0	4.1
	15	3.6	0.4	0.8	2.3
VO ₂ /NADHOX	35	38.8	3.2	11.8	37.1
	25	33.9	2.7	11.3	47.0
	15	16.7	1.4	10.6	21.9
SDH/COX	35	19.8	18.8	17.2	5.5
	25	17.8	17.3	17.1	3.84
	15	14.4	14.9	18.6	5.2
CS/COX	35	32.4	45.2	94.3	31.2
	25	44.4	56.1	103.6	36.7
	15	31.6	54.2	96.4	47.1
NADHOX/COX	35	12.0	22.7	11.3.	3.8
	25	9.1	20.3	8.9	3.2
	15	6.8	14.2	6.8	5.1
SDH/CS	35	61.5	41.6	18.3	17.5
	25	44.3	30.9	16.4	10.5
	15	46.1	27.5	19.4	11.1
NADHOX/SDH	35	60.2	120.6	65.5	68.5
	25	51.4	117.1	52.6	83.4
	15	46.9	95.4	36.7	96.8
NADHOX/CS	35	37.0	50.1	12.0	12.0
	25	22.8	34.1	8.6	8.7
	15	21.7	26.2	7.1	10.7

(All values are ratios x100)

Figure 14. Mitochondrial $\dot{V}O_2$ vs COX activity.

Figure 15. Mitochondrial VO_2 vs Scc activity.

Figure 16. Mitochondrial $\dot{V}O_2$ vs SDH activity.

Figure 17. Mitochondrial $\dot{\text{V}}\text{O}_2$ vs NADHox activity.

Figure 18. Mitochondrial VO_2 vs CS activity.

DISCUSSION

One purpose of this study was to test the hypothesis, proposed by Hoppeler and Lindstedt (1985), that the determinant of organismal $\dot{V}O_2\text{max}$ is a cellular limit to oxygen utilization by skeletal muscle mitochondria. Jones (1984) has shown that mitochondrial clustering can significantly limit mitochondrial respiration by reducing the oxygen concentration of the environment surrounding the mitochondrion. Since skeletal muscle mitochondria are very densely clustered around capillaries it is possible that oxygen availability, and not mitochondrial oxidation rate, may be the critical limit to aerobic energy production. The test of this hypothesis was to purify mitochondria from the muscle tissue and measure mitochondrial $\dot{V}O_2$ in an environment which is not oxygen-limited. By knowing what percentage of the total mitochondrial population was measured, an estimate of the aerobic potential (ml O_2 /gram-hour) of muscle tissue can be made. If the limit to $\dot{V}O_2\text{max}$ is at the cellular level, the aerobic potential of the muscle, calculated from measurements on the isolated mitochondria, should correspond with figures for whole animal $\dot{V}O_2\text{max}$. If the limit is oxygen availability, however, the calculated aerobic potential should exceed $\dot{V}O_2\text{max}$. The analysis is based on the

following assumption: 90% of $\dot{V}O_2\text{max}$ is oxygen consumed by skeletal muscle taking up approximately 45% of body mass (Weibel, 1979; Hoppeler and Lindstedt, 1985). This calculation would tend to underestimate $\dot{V}O_2\text{max}$ in animals with less than 45% muscle mass (toads) and overestimate $\dot{V}O_2\text{max}$ in animals with greater than 45% muscle mass (trout). My data show mitochondrial $\dot{V}O_2$ per gram body weight to be in excess of the published values for organismal $\dot{V}O_2\text{max}$ (Table I). Since core temperature during activity is greater than 35°C for birds and mammals, these figures can be considered an underestimate of the mitochondrial aerobic potential for endotherms. This potential is still 1.5 to 3 times larger than the published values for organismal $\dot{V}O_2\text{max}$ expressed per gram body mass. This finding indicates that estimates of $\dot{V}O_2\text{max}$ based on mitochondrial number from morphometric analysis of tissue samples may be in error because the assumption is made that all mitochondria are functioning maximally at organismal $\dot{V}O_2\text{max}$ (Hoppeler and Lindstedt, 1985). My *in vitro* results suggest that all mitochondria *in vivo* are not functioning at organismal $\dot{V}O_2\text{max}$. Jones (1984) has determined the oxygen tension necessary for the half-maximal rate of cytochrome c reoxidation, a measure of oxidative activity in mitochondria, to be about 0.1 torr. This value is well below the average physiological tissue oxygen tension of approximately 40 torr (Jones, 1984). Thus, mitochondria respire

maximally at very low oxygen concentrations, and mitochondrial $\dot{V}O_2$ can be considered to show an all-or-none response under normal physiological conditions. In addition, this data brings into question the mitochondrial $\dot{V}O_2$ value of 3-5 ml/min-ml mitochondrial volume calculated from organismal $\dot{V}O_{2\text{max}}$ data and estimates of total organismal mitochondrial number by Hoppeler and Lindstedt (1985). This does not represent the maximum potential mitochondrial $\dot{V}O_2$ based on the present *in vitro* measurements.

With decreasing temperature, mitochondria from the endothermic organisms had a significantly greater decrease in respiration when compared with the ectothermic organisms (Table I). The seven-fold higher mitochondrial $\dot{V}O_2$ per gram muscle of the endotherms at 35°C decreases significantly at 15°C, but is still two times higher than that for the the ectotherms. This agrees with earlier comparisons of mitochondrial oxidation (Lyons and Raison, 1970; Raison, et. al., 1971a,b; Hazel, 1972a,b; Roberts, et. al. 1972; Raison and McMurchie, 1974; Wodtke, 1976) which showed significantly greater decreases in rate with decreasing temperature in endotherms compared to ectotherms. The Q_{10} values for mitochondrial $\dot{V}O_2$ were significantly higher in the endotherms than in the ectotherms but comparable to previously published values for both groups (Roberts, et al, 1972; Pye, 1973). In practical terms, the

temperature at which a $\dot{V}O_2$ measurement is made can be of critical importance to an accurate measure of an organism's physiological state. It cannot be assumed that an ectotherm studied at 25°C can be compared with an endotherm studied at the same temperature, or that differences in organismal $\dot{V}O_{2\max}$ between endotherms and ectotherms can be explained on the basis of Q_{10} .

Arrhenius plots of mitochondrial $\dot{V}O_2$ differ significantly between groups, though the experimental protocol did not enable a precise determination of transition temperature. Throughout the temperature range, endotherms have significantly higher activation energies for mitochondrial $\dot{V}O_2$ than do ectotherms. Therefore, the energy required to activate mitochondrial $\dot{V}O_2$ is greater in endotherms than in ectotherms. Raison and co-workers (Lyons and Raison, 1970; Kumamoto, et al, 1971; Raison, et. al., 1971a,b; Lyons, et. al., 1974; Raison and McMurchie, 1974; McMurchie and Raison, 1979) have related this difference, through spin labeling and polarographic studies, to phase changes in the phospholipid portion of the mitochondrial inner membrane. At temperatures below 23°C the lipids in the mammalian mitochondrial inner membrane undergo a phase transition and the membrane becomes less fluid (Kumamoto, et al, 1971). This reduces diffusion rates of membrane-soluble compounds, such as ubiquinone, and may possibly increase the energy

required by allosteric enzymes to undergo the conformational changes associated with function (Kumamoto, et al, 1971; Raison, et. al., 1971a; McMurchie and Raison, 1979). Wodtke (1976, 1978,1981) and Ohira and Ohira (1988) have shown that acclimation temperature can influence both the lipid composition and transition temperature of ectotherm inner mitochondrial membranes. Other studies (Roberts, et al., 1972; Geiser et al., 1984; Raison, et. al., 1988) have shown similar changes in the composition of mitochondrial membranes of hibernating mammals, while cold acclimation had no effect on the mitochondrial membranes of non-hibernating mammals. Lyons and Raison (1970) found no difference in the temperature response of mitochondrial oxidation between warm and cold acclimated fish. My data agree with earlier comparisons of mitochondrial oxidation and $\dot{V}O_2$ activation energies between endotherms and ectotherms (Lyons and Raison, 1970; Raison, et. al., 1971a,b; Roberts, et. al., 1972; Pye, 1973; Lyons, et. al., 1974; Raison and McMurchie, 1974; Pye, et. al., 1976; McMurchie and Raison, 1978; Wodtke, 1973, 1974,1976, 1978, 1981). It is interesting to note that the high E_a for mitochondrial $\dot{V}O_2$ found in all animals is not reflected in E_a values for most of the the individually assayed enzymes. In the toads, there was no significant difference in E_a between NADHOX and mitochondrial $\dot{V}O_2$. This difference in E_a between mitochondrial $\dot{V}O_2$ and enzyme activity

may result from the presence of detergent in all enzyme assays except NADHox, where membranes were disrupted by sonication rather than detergent treatment. This suggests that modification of membrane integrity by the addition of detergents or sonic disruption alters the thermal responses of the enzyme complexes. Previous studies (Raison, et.al., 1971a; McMurchie and Raison, 1979) have shown that temperature induced phase changes in mitochondrial membranes, indicated by breaks in Arrhenius plots of mitochondrial oxidation and enzyme activity, could be removed in the presence of detergents. This suggests that it is the membrane environment surrounding the enzyme complexes, and not the complexes themselves, that is temperature sensitive. The matrix enzyme CS, which is not influenced by membrane changes, was lower in trout compared to all other animals. In all animals tested, however, the E_a for CS was lower than for $\dot{V}O_2$.

Temperature dependent changes in $\dot{V}O_2$ and enzyme activity per nmole cytochrome c+c₁ vary significantly but without relation to endothermic or ectothermic groups. Values expressed per nmole cytochrome c+c₁, based on direct spectrophotometric measurements of mitochondrial cytochromes c+c₁, are intended to quantitate individual mitochondrial characteristics with respect to concentration of an intrinsic component of the mitochondrial inner

membrane. This is necessary because mitochondrial preparations from skeletal muscle contain varying amounts of non-mitochondrial protein which are not distinguishable from mitochondrial protein using standard protein assay techniques. In addition, activities expressed per gram wet weight tissue often obscure species specific differences in mitochondria. This is important in light of the very low $\dot{V}O_2$ per nmole c+c₁ for bird mitochondria compared to mammal mitochondria even though the two are very similar on a per gram muscle basis. Amphibians and fish represent a similar case in point: $\dot{V}O_2$ per nmole cytochrome c+c₁ is substantially higher in fish than in amphibians. Enzyme activities expressed in the same units (Table IIb) show no definite pattern uniting all mitochondria. Each vertebrate class has its own mitochondrial enzyme profile and characteristic responses to temperature.

Ratios of mitochondrial enzyme activity were compared to determine if there were common relationships between animals not represented by absolute activities (Table IV). While the ratios of NADHOX to CS, SDH to COX, CS to COX and SDH to CS activity were significantly higher in endotherms compared to ectotherms, there were no cases where ratios were the same between species within a group. In particular, while the ratio of SDH to COX differed significantly between endotherms and ectotherms, mammals, birds

and amphibians were not significantly different. The remaining activity ratios show no apparent relationship to vertebrate class or endothermic or ectothermic group. Of note is the three-fold greater ratio of CS to COX in amphibians and the two-fold greater ratio of NADHOX to SDH in birds compared to the other animals.

CONCLUSIONS

The above data indicate that mitochondria are different in species representing four vertebrate classes. Raison, et. al. (1988) have shown that mitochondria have tissue specific properties in a hibernation adapted mammal and Roberts, et. al. (1972) have shown species specific differences in mitochondrial succinoxidase (ETS complexes II,III and IV) activity between hibernating and non-hibernating mammals. Since differences in mitochondrial physiology can be found within species belonging to the same vertebrate class, differences in mitochondrial physiology between classes are not unexpected. While the present study is limited by the number of species studied, it is clear that all vertebrate muscle mitochondria are not the same. This conclusion strongly speaks to the need for great care in comparative analyses based on single animal studies. Additionally, enzymatic measures intended to estimate aerobic capacity are not applicable across taxonomic lines. While analyses of cellular and organismal $\dot{V}O_2$ based solely on mitochondrial numbers are important to our understanding of aerobic physiology, they may lead to incorrect conclusions due to lack of attention to mitochondrial variation. My data show that mitochondria *in vitro* are able to respire at rates much higher than

are estimated from measures of organismal $\dot{V}O_{2\max}$. This indicates, therefore, that the limit to organismal $\dot{V}O_{2\max}$ must lie somewhere other than in the mitochondrion. It is apparent that more effort is needed to characterize mitochondrial physiology and biochemistry for a wide range of experimental animals.

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APPENDIX

ASSAYS OF THE MITOCHONDRIAL ELECTRON TRANSPORT SYSTEM

STOCK SOLUTIONS

1) 0.25M Potassium phosphate buffer (pH=7.4). Monobasic potassium phosphate (KH_2PO_4) MW= 136.09, dissolve 3.4g in 80ml double distilled water (ddH₂O) and adjust pH to 7.4 with 5N KOH. Dilute to 100ml final volume with ddH₂O. May be stored refrigerated for 1 month.

2) 0.5M Phosphate/5% Tween 80 buffer (10x P/T buffer). Dissolve 6.8g KH_2PO_4 in 80ml ddH₂O, add 5ml Tween 80 and stir until detergent dissolves. Adjust pH to 7.4 with 5N KOH and dilute to 100ml final volume with ddH₂O. May be stored refrigerated for 1 month.

3) 1x Phosphate/Tween buffer (P/T buffer), pH 7.0. Dilute 10ml 10xP/T buffer with 70ml ddH₂O and adjust pH to 7.0 with 1N HCl. Dilute to 100ml final volume with ddH₂O. May be stored refrigerated for not more than one month.

4) 0.05M Magnesium Chloride (MgCl_2). MgCl_2 hexahydrate $\text{mw}=203.33$. Dissolve 1.02g into 100ml final volume ddH₂O. May be stored refrigerated for 1 month.

5) 0.2M Potassium cyanide (pH 7.4). KCN $\text{mw}= 65.12$. Dissolve 0.13g into 5ml ice cold ddH₂O in an ice bath in a fume hood. Adjust pH to 7.4 with ice cold 1N HCl(about 1.5ml) using narrow range pH paper. Dilute to 10ml final volume with ddH₂O, cover tightly and keep on ice or under refrigeration until used. Make fresh daily.

6) 1.0M Succinate (pH 7.4). Succinic acid $\text{mw}=118.1$. Dissolve 1.18g in 7ml ddH₂O and adjust pH to 7.4 with 5N KOH. Dilute to 10ml final volume with ddH₂O. One ml aliquots may be stored at -70°C for up to one year.

7) 1mM Ferrocyclochrome c, $\text{mw}=12,384$. A P6DG (BioRad polyacrylamide) desalting column (0.7X 20cm) is equilibrated with 50mM phosphate buffer (pH 7.0). 25mg cytochrome c (Sigma horse heart type IV) is dissolved in 1ml 50mM phosphate buffer and reduced by addition of 50 μl 0.5M ascorbate with 1mM EDTA (pH5). The reduced "c" solution is then added to the P6DG column and eluted with 50mM phosphate buffer (pH 7). The dark red band is collected and the dilute tail left on the column. The concentration of

ferrocytochrome c is determined as described below and diluted, if necessary, to 1 mM with phosphate buffer. Aliquots of 0.2ml may be stored frozen at -70°C for up to one year.

8) 1 mM Ferricytochrome c, mw= 12,384. Dissolve 13mg into 1ml 50mM phosphate buffer (pH 7.4). Make fresh daily.

9) 2mg/ml Rotenone (approximately 5mM). mw= 394.1. Dissolve 10mg in 5ml 100% ethanol in brown glass, stir until dissolved. Rotenone is extremely light sensitive and should be stored, weighed and prepared in subdued light and brown glass. Make rotenone solutions fresh daily.

10) 2mM 2,6-Dichlorophenol indophenol (DCIP) mw= 291.0. Dissolve 5.8mg into 10ml final volume with ddH₂O. Make fresh daily and store in brown glass.

11) 40mM Phenazine methosulfate (PMS) mw=310. Protect from light during weighing, pipetting , and at all times. Dissolve 12.4mg into 1ml ddH₂O . This solution is extremely light sensitive: make fresh daily and store in brown glass.

12) 1mM Potassium ferricyanide $\text{K}_3\text{Fe}(\text{CN})_6$ mw=329.6. Dissolve 33mg into 100ml final volume ddH₂O. Make fresh daily.

NADH OXIDASE

This assay follows the oxidation of NADH at 340nm by Complexes I, III, and IV (NADH:Oxidase) of the electron transport system.

A working buffer is made, fresh daily, from the following (20 assays):

<u>Solution</u>	<u>Amount</u>	<u>Final Concentration</u>
1) 0.25M Phosphate buffer(pH 7)	1ml	25mM
2) 0.05M MgCl ₂	1ml	5mM
3) 1mM Ferricytochrome	0.1ml	10μM
4) NADH	1.6mg	0.2mM
5) BSA	25mg	2.5mg/ml
6) ddH ₂ O	<u>7.4ml</u>	
Final Volume	9.5ml	

To a 1.5ml Eppendorf tube add:

7) 475μl Working buffer

8) 25μl Mitochondrial suspension 50μg/ml

Sonicate in ice bath 3x15 seconds at setting 1 (about 30 watts on a Branson128 sonicator). Place sonicated sample in a prewarmed 0.6ml cuvette and measure change in OD ₃₄₀ . Add 1µl rotenone solution to the sample after 2min and read for another 3min. This activity in the presence of rotenone is subtracted from the total activity to determine the electron transport chain activity.

Calculations: The NADH:Oxidase assay measures the decrease in absorbance of NADH at 340nm. A line is drawn through the steepest linear part of the trace (the rate is usually linear for more than three minutes) and the initial and final absorbance are measured, the difference in initial minus final absorbance = ΔA . The calculation of specific activity is as follows:

$$\Delta A / (6.22 \times 5 \times 0.05) = \text{specific activity } (\mu\text{M}/\text{min}/\text{mg}),$$

where 6.22 is the millimolar extinction coefficient for NADH.

REFERENCE: Fischer, et. al. (1986)

SUCCINATE CYTOCHROME C REDUCTASE AND SUCCINATE DEHYDROGENASE

These assays measure the activity of Electron Transport Complexes II and III by following the reduction of ferricytochrome c or dichlorophenol-indophenol (DCIP) by succinate. The Succinate Dehydrogenase (SDH) assay measures the activity of the two subunits of Complex II comprising SDH using the artificial electron acceptor phenazine methosulfate (PMS) as primary electron acceptor, then donating electrons to DCIP. Substitution of ferricytochrome c for PMS and DCIP and reading absorbance at 550nm gives a measure of the combined activity of Complexes II and III (Succinate: Cytochrome c reductase).

An assay buffer is made, fresh daily, of the following (20 assays):

<u>Solution</u>	<u>Amount</u>	<u>Final Concentration</u>
1) 0.25M Phosphate buffer (pH 7.4)	2ml	50mM
2) 1.0M Succinate (pH 7.4)	0.2ml	20mM
3) 0.2mM KCN (pH 7.4)	0.1ml	2mM
4) 2mg/ml Rotenone	10µl	2µg/ml

5) ddH₂O 6.99ml

Final Volume 9.30ml

Pre-incubation: (to activate samples prior to assay)

The following is incubated in a 30° water bath for 30 minutes:

6) 10x Phosphate/Tween	10μl	50mM/0.5%
7) 1.0M Succinate	2μl	20mM
8) Diluted mitochondria 1:20*		50μl
9) ddH ₂ O		<u>38μl</u>
Final volume		100μl

After incubation keep on ice until used.

* A 10μl aliquot of concentrated mitochondria is diluted to 200μl with SET with 0.1% Tween 80 (1:20).

SET= 250mM Sucrose, 10mM Tris HCl, 2mM EDTA, 0.1% Tween 80, pH 7.4.

Assay Procedure:

To a 0.6ml cuvette add:

1) 465μl Assay Buffer

2) 10 μ l activated mitochondria (final Tween concentration=0.01%)

Place cuvette in spectrophotometer and allow 2 minutes to warm to 30°.

For SDH: Add 12.5 μ l each of 40mM PMS and 2mM DCIP and mix as above. Record decrease OD₆₀₀ for 3 min. The final concentration of PMS is 1mM, the final concentration of DCIP is 50 μ M.

For Succinate: cytochrome c reductase: Add 25 μ l ferricytochrome c, mix well by inverting 3x and replace in the spec. Record increase OD₅₅₀ for 3 min. The final concentration of ferricytochrome c is 50 μ M.

The millimolar extinction coefficient for reduced cytochrome c at 550nm is 27.6. Specific activity is determined as follows:

$$\Delta A / (27.6 \times 3\text{min} \times [\text{protein}]\text{mg})$$

The millimolar extinction coefficient for reduced DCIP at 600nm is 19.1. Specific activity is calculated as follows:

$$\Delta A / (19.1 \times 3\text{min} \times [\text{protein}]\text{mg})$$

REFERENCES: Fischer, et. al. (1985), Singer, T.P. (1974)

CYTOCHROME C OXIDASE

This assay follows the oxidation of ferrocytochrome c by cytochrome oxidase by following the decrease in OD₅₅₀.

Solutions:

- 1) 50mM Phosphate/0.5% Tween buffer, pH 7.0
- 2) 1mM Ferrocytochrome c
- 3) Diluted mitochondria (1:20 in SET/0.1% Tween 80)
- 4) 1mM Potassium ferricyanide

Determination of [ferrocytochrome c]:

Because the rate of reaction is directly dependent upon the concentration of ferrocytochrome c, an assay of the stock solution is conducted just prior to the COX assay.

To a 0.6ml cuvette add:

- 5) 495µl Phosphate/Tween buffer
- 6) 5µl ferrocytochrome c stock
  - a) read OD₅₅₀
  - b) add a small grain of sodium dithionite and mix well
  - c) read OD₅₅₀ (absorbance of 100% reduced solution).

d) divide (a) by (c) to determine the percent reduced ferrocytochrome c in the stock solution. The solution should be greater than 90% reduced or discarded. The concentration of ferrocytochrome c in the stock solution may be determined by dividing the absorbance (a) by 0.276, this yields the concentration of ferrocytochrome c (μM).

Cytochrome c Oxidase Assay

To a 0.6ml cuvette add:

- 7) 485 μl 1x Phosphate/Tween buffer
- 8) 10 μl Mitochondrial suspension

Place in spectrophotometer and allow 5 min incubation at 30°C.

- 9) Quickly add 5 μl reduced c, immediately start spectrophotometer or stopwatch, mix cuvette by inverting 3x and replace in spec.

This sequence is essential to determine the initial rate of cytochrome oxidase activity.

- 10) Record decrease OD₅₅₀ for 5 minutes, accumulating at least 10 data points (30 second intervals). Then add 1 μl ferricyanide, allow 1min for sample to react and record the fully oxidized

OD₅₅₀. This oxidized value is subtracted from all previous data points.

The spectrophotometric assay of Cytochrome c Oxidase follows first order reaction kinetics which are directly proportional to the initial concentration of ferrocytochrome c. It is essential that the initial concentration of ferrocytochrome c remain constant for all assays. The rate falls off as an exponential function of [ferrocytochrome c] which is described by the equation:

$$v_t = v_0 e^{-kt}$$

To calculate Cytochrome Oxidase activity:

1) Plot the data points on semi-log graph paper and determine the slope.

a) Rate constant (k) = slope $\times 2.303$ (this converts from $\log_{10}$ to e).

b) Initial velocity (v_0) = $k \times [\text{ferrocytochrome c}]_0$; the units are nmol/ml/min.

c) Specific activity = $v_0 / [\text{mitochondrial protein per ml assay}]$; the units are nmol/min/mg.

2) Alternatively, k can be determined by calculating the half time ($t_{1/2}$) of the reaction: $k = 0.693 / (t_{1/2})$.

3) A third alternative is to calculate k by means of an exponential curve fitting program .

Note: This assay is optimal at $10\mu\text{g/ml}$ human mitochondrial protein and $1\mu\text{g/ml}$ for beef heart and muscle. Care must be taken to ensure that the reaction does not proceed too rapidly or accuracy is reduced. The optimal value for k is between 0.2 and 0.5, and the concentration of protein should be adjusted , by diluting sample, to give an activity within this range.

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