## ABSTRACT

Synthesis of ATP in eukaryotic creatures is controlled by several mechanisms including glycolysis, the Krebs cycle, and the electron transport system (ETS). ETS is located on the inner mitochondrial membrane and can be readily analyzed for function *in vitro*. Here, we report on the suitability of rat liver mitochondria as a model for studying, specifically, the ETS, and mitochondrial function as a whole. Isolated mitochondria were treated with glutamate and succinate; the poisons antimycin, rotenone, KCN, FCCP, and oligomycin; and the artificial electron donor combination of ascorbate and TMPD to evaluate the form and function of ETS. ADP:O ratios were calculated to assist in the deduction of action sites for various substrates. State III respiration on succinate yielded an ADP:O ratio of 1.9:1 and 3.3:1 on glutamate, indicating two ETS points of entry at complexes II and I, respectively. The state IV rate of respiration on glutamate was three times slower than on succinate. Rotenone was found to competitively bind complex I and the sites-of-action for antimycin and TMPD were narrowed considerably. KCN inhibited respiration on glutamate completely. FCCP uncoupled the ETS from oxidative phosphorylation and oligomycin was responsible for inhibiting the latter. The breadth of data obtained supports the belief that rat liver mitochondria are an extremely suitable model for the study of both specific and generalized mitochondria function.

# INTRODUCTION

Adenosine triphosphate (ATP) is a multifunctional cellular compound critical to the continued existence of all known life. It both stores energy obtained from various substances and supplies it for use in cellular processes. One of the mechanisms critical to the synthesis of ATP by eukaryotic cells is the maintenance of a proton-based chemiosmotic gradient within mitochondria by the Electron Transport System (ETS). This study evaluated the suitability of rat liver mitochondria as a model for studying mitochondrial function and specifically, ETS. Understanding ETS's function is critical to understanding an integral part of the mechanisms by which cells obtain energy and thereby stay alive.

Rat liver was chosen as a source of mitochondria from the practical standpoint because 1) laboratory rats are readily obtainable, 2) homogenization of animal tissue is easier than cell wall-covered plant cells, and 3) and lesser amounts of connective tissue in liver make obtaining substantial yields of mitochondria a relatively quick process. Furthermore, in theory, it can be expected that mitochondria yields will be higher from endotherms because they require more mitochondria to allow for higher metabolism to generate heat.

As electron transport is both a key function of mitochondria and a critical cellular process, questions arise as to how the system itself functions. This requires an understanding of the function of the individual enzyme and protein complexes within the system. Thus, we observed *in vitro* the effects on both disrupted and uninhibited cellular respiration by various substrates and metabolic poisons at different steps in the ETS process. More specifically, this study questioned where these substrates and poisons affected the transport chain.

We isolated mitochondria from fresh rat liver using differential centrifugation. Changes in cellular respiration were then observed through use of an oxygraph by adding enzyme

complex-specific substrates and metabolic poisons to ADP-stimulated mitochondria. Analysis of the consumption of oxygen due to the addition various substrate/poison combinations will reveal their binding locations, the characteristics of electron movement through the chain, and the purpose of the chemiosmotic gradient as the method of respiratory control.

# MATERIALS AND METHODS

## Mitochondria Preparation

Rats weighing 200-250 g were killed and immediately dissected, revealing the liver. Chilling was started immediately by pouring a generous amount (100 mL) of 0.9% NaCl into the peritoneal cavity. The liver was then removed to a cold saline solution of the same composition. It was then divided into two equal 3-5 g portions after which the saline solution was poured off so liver could be minced "dry." After mincing, 20 ml of homogenizing solution was added to the liver. The solution was composed of 0.25 M sucrose, 5 mM HEPES buffer, and 1 mM EDTA, pH 7.2. We used a “Tissuemizer” with a T25 stainless steel shaft made by Tekmar (Cincinnati, OH) at setting ’40’ for 10 s to homogenize the liver mince. The homogenate was then transferred to a centrifuge tube, topped off with homogenizing solution, and spun down at 500 x g for 10 m at 4°C. The supernatant was then spun down in a clean tube at 9400 x g for 10 m, also at 4°C to concentrate the mitochondria into pellet form. The supernatant was carefully removed, and the pellet was mixed to a paste, moved to an eppendorf tube, and kept on ice.

## Calibration of Oxygraph and Chart Recorder

We used custom-made Gilson-type glass chambers fitted with a #5331 standard oxygen probe and Yellow Springs Instruments 5300 Oxygen Monitors. We also used Zipp and Konen chart recorders to graphically record oxygen consumption. The respiration medium consisted of 70 mM sucrose, 220 mM mannitol, 2 mM HEPES buffer, 5 mM MgCl, 5 mM $K_3PO_4$, and 0.1% fatty-acid-free bovine serum albumin, pH 7.4.

The empty respiration chamber was rinsed thoroughly with deionized water and then filled with 2.2 mL of respiration medium and allowed to equilibrate unstoppered. The oxygen

monitors and chart recorders were set to full scale (100% = full oxygen saturation) and calibrated accordingly.

**Experiments on Rat-Liver Mitochondria**

The amount of respiration medium added to the chamber was noted prior to each experiment. Substrates and metabolic poisons were added to the chamber once steady states of respiration (state III or IV) were observed unless otherwise noted. All mitochondria were micropipetted into the chamber after which the chamber was immediately stoppered. Subsequent additions of substrates, metabolic poisons and ADP were added through the stopper by a Hamilton syringe.

Fifteen µL of mitochondria were added to the respiration chamber along with 20 µL of 0.5 M succinate to cause state IV respiration on succinate. Thirty µL of mitochondria were used in conjunction with 20 µL of 0.5 M glutamate for state IV respiration on glutamate. Stimulation of state III respiration was achieved with 20 µL of 0.01 M ADP. Addition of further substrates or poisons was postponed until state IV respiration resumed. Electrons were artificially donated to the ETS through the use of 10 µL of 30 mM TMPD and 0.5 M ascorbate in combination. To inhibit the ETS, 10 µL of 10 mM antimycin were added. We also utilized rotenone in the same fashion. KCN was used in 10 µL, 0.5 M amounts to inhibit respiration as well. Uncoupling was obtained through the addition of 10 µL of 10 mM FCCP (carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone) while 10 µL of 10 mM oligomycin were added to inhibit oxidative phosphorylation. With the exception of KCN, all poisons were prepared in absolute ethanol. Aqueous solutions of TMPD, ascorbate, KCN, and the substrates were prepared at pH 7.

# RESULTS

## Mitochondria Yield

Volume measurements of mitochondria yield revealed for possible future studies that a 12.6 g liver produced a little over 1.5 mL of concentrated mitochondria.

## Respiration Studies

In each experiment, a short, rapid depletion of oxygen was initially observed. A slower but continuous consumption of oxygen began thereafter until the addition of the first substrate or poison.

*Respiratory Control on Succinate*

After the injection of succinate, a steady state IV respiration was observed (Table 1). After the addition of ADP, state III respiration commenced until all the ADP was phosphorylated. The change in respiration rates from 2.8 X $10^{-3}$ to 1.3 X $10^{-3}$ µmoles $O_2$ consumed per min per µL mitochondria indicated a shift back to state IV respiration (Table 1). The ratio of the moles of ADP phosphorylated to atoms of oxygen consumed on succinate was 1.9:1 (Table 5). An increase in $O_2$ in the chamber was observed immediately after the addition of antimycin to the chamber and decreased the rate of respiration considerably. Adding TMPD and ascorbate caused a rapid increase in oxygen consumption (Table 1).

**Table 1**. Rates of Respiration on Succinate
(2.2 mL respiration medium; 15 µL mitochondria)

| Reagent | Rate (µmoles $O_2$/min/mL mitochondria) |
|---|---|
| 20 µL 0.5 M succinate | 9.9 X $10^{-4}$ |
| 20 µL 0.01 M ADP (III)[1] | 2.8 X $10^{-3}$ |
| (IV)[1] | 1.3 X $10^{-3}$ |
| 10 µL 10 mM antimycin | 1.5 X $10^{-4}$ |
| 10 µL 0.5 M ascorbate & 10 µL of 30 mM TMPD | 2.2 X $10^{-3}$ |

[1] state of respiration on ADP

*Respiratory Control on Glutamate*

State IV respiration on glutamate was over three times slower than state IV respiration on succinate (Table 2), and the ratio of ADP's phosphorylated per oxygen atom consumed during state III was 3.3:1 (Table 5). This continued until state IV resumed. The addition of rotenone slowed respiration by over 5 times its previous rate. *No change in rate of oxygen consumption occurred upon the addition of ADP* (Table 2). However, when succinate was added, two distinct respiration rates (state III and IV) were observed. The ADP:O ratio was also 1.9:1 as in the first study (Table 5). The addition of KCN halted respiration almost completely (Table 2).

*Uncoupling Agent Effects on Respiration*

Data for this portion of the study was obtained from Michelle Afkhami, Whitney McAlpine and James Stone. State IV respiration rate on succinate similar to those of the other studies was observed. The addition of FCCP yielded an 8-fold increase in oxygen consumption (Table 3).

**Table 2**. Rates of Respiration on Glutamate and Succinate, Poisoned and Unpoisoned
(2.2 mL respiration medium; 30 µL mitochondria)

| Reagent | Rate[2] |
|---|---|
| 20 µL 0.5 M glutamate | $2.8 \times 10^{-4}$ |
| 20 µL 0.01 M ADP (III)[1] | $1.6 \times 10^{-3}$ |
| (IV)[1] | $7.4 \times 10^{-4}$ |
| 10 µL 10 mM rotenone | $1.3 \times 10^{-4}$ |
| 20 µL 0.01 M ADP | $1.3 \times 10^{-4}$ |
| 20 µL 0.5 M succinate (III)[1] | $2.2 \times 10^{-3}$ |
| (IV)[1] | $1.2 \times 10^{-3}$ |
| 10 µL 0.5 M KCN | $7.4 \times 10^{-5}$ |

[1] state of respiration on ADP
[2] rate units same as in Table 1

**Table 3**. Effect of Uncoupling Agent FCCP on Succinate-based Respiratory Control
(2.2 mL respiration medium; 15 µL mitochondria)

| Reagent | Rate[1] |
|---|---|
| 20 µL 0.5 M succinate | $4.0 \times 10^{-4}$ |
| 10 µL 10 mM FCCP | $3.5 \times 10^{-3}$ |

[1] rate units same as in Table 1

*Inhibition of Oxidative Phosphorylation*

We again observed the previously noted effects on oxygen consumption by starting respiration on succinate. Addition of oligomycin decreased the rate of respiration to $8.7 \times 10^{-4}$ µmoles $O_2$ per min per µL of mitochondria (Table 4). Almost no change in rate of respiration was observed despite the addition of ADP in the presence of a substrate. The addition of FCCP commenced rapid oxygen consumption, but no distinct changes in $O_2$ consumption were seen.

**Table 4**. Respiration Rate of Oligomycin- and FCCP- treated Mitochondria on Succinate
(2.2 mL respiration medium; 15 µL mitochondria)

| Reagent | Rate[1] |
|---|---|
| 20 µL 0.5 M succinate | $1.3 \times 10^{-3}$ |
| 10 µL 10 mM oligomycin | $8.7 \times 10^{-4}$ |
| 20 µL 0.01 M ADP | $7.7 \times 10^{-4}$ |
| 10 µL 10 mM FCCP | $4.2 \times 10^{-3}$ |

[1] rate units same as in Table 1

**Table 5**. ADP:O Ratios for ETS Substrates

| | |
|---|---|
| Experiment 1 (on succinate) | 1.9 |
| Experiment 2 (on glutamate) | 3.3 |
| (on succinate) | 1.9 |

# DISCUSSION

## Respiration Studies

### *Respiratory Control on Succinate*

The initial depletion of oxygen after the addition of mitochondria is likely the result of oxygen binding to the unoccupied sites in mitochondria, most importantly, complex IV of the ETS where any left over substrates from the live animal were processed immediately through the system and oxygen was reduced to water. Yet, after the initial loss of oxygen, a slower consumption continued due to the probability that the ETS was consuming the fatty-acid substrates found in the tissues that were homogenized.

The addition of substrate produced state IV respiration with an observable rate of oxygen consumption (Table 1). Respiratory control does not completely eliminate state IV expression because mitochondria have other functions (i.e. symport of inorganic phosphate with gradient protons) that exploit the energy stored in the chemiosmotic gradient. Furthermore, homogenization likely damages the outer membrane of some mitochondria, therefore, *in vitro* only, a gradient cannot be maintained and mitochondria cannot control respiration. *In vivo*, cell processes eliminate damaged and/or faulty mitochondria, so this *in vitro* expression does not occur. However, since the gradient is relieved in intact mitochondria by other mechanisms, succinate nevertheless *is* oxidized to fumarate during state IV respiration. The electron pair from succinate is received directly at an ETS entry complex and is processed through coenzyme Q, an energy carrier; complex III, which translocates protons; cytochrome C, another energy carrying protein; and finally through complex IV, which translocates more protons and binds and reduces oxygen to water. The slow state IV dissipation of the chemiosmotic gradient permits any ETS

substrate to yield the reduction of oxygen at a certain rate; therefore, the rate-limiting step can be determined to be non-ADP-phosphorylation-related dissipation of the chemiosmotic gradient.

The addition of ADP, however, increases respiration by binding along with an inorganic phosphate to ATP synthase, a membrane enzyme, and opening a pathway for protons to flow back into the mitochondrial matrix, thereby relieving the gradient and permitting faster cellular respiration. This reaction phosphorylates ADP and turns it into ATP. Less oxygen is needed by glutamate to maintain the chemiosmotic gradient during state III respiration (it translocates more protons per electron pair) than by succinate (Table 1). Therefore, the substrate is the rate-limiting factor in this case since the maintenance of the gradient energy level during usage by ATP synthase is dependant upon the efficiency of its replenishment. A comparison of ADP:O ratios demonstrates this. The ratio of glutamate (3.3:1) as compared to succinate (1.9:1) suggests that more ADP's are phosphorylated with each oxygen atom. This requires more free energy to be moved to the gradient to maintain the balance of the chemiosmotic gradient which results in faster replenishment of the gradient.

While *in vivo*-Krebs cycle intermediates *will* affect ADP:O ratios, they are not to be considered relevant *in vitro* because the Krebs reaction rates are dependant on the concentration of the previous reactant. The reactant concentrations added in this experiment were *much* higher than the product (virtually none) therefore rendering the latter negligible.

After all the ADP is phosphorylated, however, the state IV respiration that resumed was faster than the first state IV rate for both substrates (Table 1). One possible explanation for this theory is the possibility of actual uncoupling effects of ADP itself. *In vivo,* ATP-deprived cells soon experience damaged membranes due to a high amount of unphosphorylated ADP, and a damaged mitochondria membrane in some mitochondria yields an increased rate of respiration.

Inundating mitochondria with ADP *in vitro* simulates this ATP-starvation effect. Nevertheless, it is important to remember that state III and IV respiration do not exist *in vivo* since many products and intermediates in various cell processes serve multiple purposes and can be siphoned off the process for use or introduced at an intermediary stage.

*Respiratory Control on Glutamate*

Thirty µL of mitochondria was used to enhance visualization due to the slower respiration rate of glutamate (Table 2). It therefore becomes necessary to standardize respiration rates by the µL of mitochondria when rates of varying volumes must be compared because more mitochondria have more binding sites for oxygen consumption and will consume it faster.

But glutamate itself is neither a Krebs intermediate nor an ETS substrate. Its oxidation yields NADH which is then oxidized at complex I with the energy transfer stored by proton translocation. Thereafter, it binds to coenzyme Q and follows the same path as succinate. The slower state IV respiration rates and higher ADP:O ratios are discussed above.

Theoretically an integer, the ADP:O ratio represents the number of ADP's produced by the consumption of one oxygen atom during respiration. However, since the chemiosmotic gradient serves multiple functions and/or some mitochondria could be damaged as previously discussed, the ratio, experimentally, is different.

The addition of rotenone, however, did not halt state IV respiration completely. This suggests that the ETS still functions and rotenone therefore only competitively inhibits respiration. This, however, makes it the rate-limiting step so the addition of ADP does not cause an increase in respiration. Yet, that succinate *did* go through state III and IV respiration suggests that rotenone binds to a complex that inhibits respiratory control on glutamate only, before the paths from complex I and II converge. Rotenone, we can conclude, binds competitively to

complex I. Thus, antimycin must inhibit ETS at complex II, coenzyme Q, Complex III, or cytochrome C since TMPD and ascorbate must donate their electrons at least at Complex IV. Future studies can narrow the location by testing antimycin's effect on glutamate and TMPD's effect on cytochrome C or complex IV.

**Uncoupling Agents**

The rapid consumption of oxygen (Table 3) suggests that respiratory control was lost. Called uncoupling, the dissolution of the chemiosmotic gradient and separation of ATP synthase from the ETS was carried out by FCCP, an ionophore. The respiration rate is much faster than state III respiration, and the rate-limiting factor in uncontrolled respiration is the rate of physical interaction between complexes floating within the lipid membrane. While neither ADP nor a different substrate affects the rate of respiration (Table 4), a transport inhibitor does because it blocks the pathway to reducing oxygen in its entirety, so no respiration can occur. ADP cannot affect respiration because no gradient is present to be relieved by phosphorylation. The inability of mitochondria to phosphorylate ADP is the *very essence* of uncoupling. Nevertheless, *substrate* consumption still takes place because the ETS must have a fuel for its uncontrolled respiration. The energy is continually expended as heat. In fact, brown fat mitochondria in some endotherms have a similar task of generating heat through a controlled form of uncoupled respiration. *In vivo*, a source of fuel is required to continue facilitating uncontrolled respiration, and thus, carbohydrates, and fats, etc. are turned into substrates. Next, muscles are victimized, and soon enough, the individual is dead. This makes uncoupling a very dangerous task.

**Inhibition of Oxidative Phosphorylation**

The rates of respiration observed during the addition of ADP with substrate present after oligomycin suggest that oligomycin blocks ATP synthase from functioning. The addition of FCCP after ADP uncoupled the reaction as before. If oligomycin were an ETS inhibitor, oxygen consumption would have halted, but this was not the case. By deduction, oligomycin must then inhibit oxidative phosphorylation.

Clearly, rat liver mitochondria are a suitable model for the study of the electron transport system and, more generally, mitochondria itself. Calculable respiration rates and clear indicators of respiration alteration suggest that rat liver mitochondria responds well to treatment with various substrates or metabolic poisons. If future studies are conducted, researchers will be able to pinpoint the exact binding sites and functionality of chemicals such as antimycin, TMPD/ascorbate, and others as well as gain a better understanding of the purposes of the chemiosmotic gradient and how it factors into cellular activity and thus, the life of the organism.