STUDIES ON THE COUPLING OF MITOCHONDRIAL ELECTRON TRANSPORT BY PROTON MOTIVE FORCE TO ATP SYNTHESIS

According to the chemiosmotic theory, the oxidation of respiratory substrates, phosphorylation of ADP to ATP as well as ion transport into and from mitochondria, are coupled via a proton concentration gradient as the common intermediate for all these processes. Gibbs' free energy generated by redox reactions of the respiratory chain provides the driving force to transport H^+ ions from the matrix (mx) to the intermembrane space of mitochondria (im), forming a concentration gradient for H^+ ions and electrical potential difference (ΔE_m) caused by charge separation to the two faces of the inner membrane. The sum of the two energies is called proton motive force (proton electrochemical potential):

$$\Delta P = \Delta E_m + \frac{2.3RT}{F} \lg \frac{[H^+]_{im}}{[H^+]_{mx}} = \Delta E_m - 0.06(pH_{im} - pH_{mx}) = \Delta E_m - 0.06\Delta pH$$

Active transport processes such as the uptake of Ca^{2+}, phosphate, succinate, newly synthesized mitochondrial proteins, etc. from cytoplasm, and also the phosphorylation of ADP from ADP and inorganic phosphate are built on the use of the energy of proton motive force. In the phosphorylation of 1 mole ADP to ATP 2 moles of H^+ ions are redistributed from the intermembrane space to the matrix. Substances destroying proton concentration gradient remove the driving force for ATP synthesis and active transport processes; therefore, the coupling between the reactions of producing and using energy will be lost. These compounds - called uncouplers - are usually lipophilic substances with proton binding ability at around pH 6.5 (weak acids), such as 2,4-dinitrophenol.

Carefully chosen conditions (ionic constituents of medium) permit the study of how proton motive force is used to transport ions across the inner membrane of mitochondria, or to phosphorylate ADP to ATP. The coupling between electron transport and phosphorylation in our experiments will be studied by recording oxygen uptake of mitochondria suspension in the presence of respiratory substrates, inorganic phosphate, ADP and various effectors.

OXIDATION OF SUBSTRATES AND ATP GENERATION

1. Respiratory control and P/O ratio

In conditions where intact mitochondria (while phosphorylating ADP to ATP) oxidize respiratory substrates**,** both the rate of oxygen consumption and the amount of ATP synthesized in the process can be assayed. The rate of substrate oxidation at optimum conditions (saturating concentration of respiratory substrate, inorganic phosphate, oxygen dissolved in buffered isotonic medium, at neutral pH) depends only on the presence of ADP. The accelerating effect of ADP on substrate oxidation is called **acceptor control**, or **respiratory control**: in the absence of ADP the rate of respiration is low (resting respiration), but becomes high if ADP, even in low concentrations, is added. After ADP has become completely phosphorylated, oxygen uptake returns to a low rate. The proportion of the rate of oxygen uptake measured in the presence of ADP ($v_{(+ADP)}$) to the rate measured after ADP has been

used up ($v_{(-ADP)}$) is characteristic of the efficiency of coupling of the respiration and ATP synthesis, and is called **respiratory control ratio**:

$$\mathbf{RC} = \frac{v_{(+ADP)}}{v_{(-ADP)}} > 1$$

If the incorporation of inorganic [^{32}P]-phosphate into ATP and the uptake of oxygen are simultaneously recorded, the stoichiometric ratio of ATP synthesized during substrate oxidation can be calculated as*:*

$$\mathbf{P/O} = \frac{\text{mole } [^{32}\text{P}]\text{-ATP}}{\text{mole oxygen atom}} > 0$$

in case of intact mitochondria, its actual value depends on the site where the respiratory substrate enters the electron transporting chain (additionally, in some cases phosphorylation also occurs on 'substrate level').

If any uncoupler is present, or the inner membrane is leaky, both the **RC** and **P/O** decrease, thus ADP fails to accelerate respiration, and no net ATP is synthesized, although the rate of oxygen uptake is still high.

If, in well-coupled mitochondria, the synthesis of ATP is inhibited by the antibiotic **oligomycin**, respiration also becomes suppressed, because proton motive force generated by respiration reaches a limit represented by the Gibbs' free energy change of substrate oxidation (and*,* also, the matrix becomes alkaline). A similar effect can be expected if a lack of phosphate, or ADP occurs. Transport of ADP from cytoplasm to the matrix side through **ADP:ATP translocase** of inner mitochondrial membrane can be inhibited by **atractylosides**, also inhibiting respiration, although proton motive force remains still high. In these cases respiration restarts if uncoupler is added or ion transport (e.g. Ca^{2+} uptake) is initiated.

2. Inhibiting respiratory chain.

Substances known as **site-specific inhibitors** of respiration decrease oxygen uptake, and they are used when studying the nature of sites oxidizing a given substrate. Due to the lack of energy production, neither the generation of proton motive force, nor net synthesis of ATP (or active ion transport) can be expected, and respiration will not restart*,* **even if ADP or an uncoupler is added**. In the presence of a given inhibitor of the electron transport chain*,* all components of the chain between the used substrate and the site of action of inhibitor become reduced, while all following components toward oxygen become oxidized. This phenomenon can be demonstrated by comparing redox spectra of electron transport components of mitochondria in the absence and presence of the inhibitor. The most important respiratory inhibitors are: **CN^-**, **CO**, **N_3^-** (inhibiting at cytochrome oxidase), **antimycin A** (between cytochrome b and c_1), **malonate** (at succinate dehydrogenase) and **rotenone** (at NADH-dehydrogenase).

3. Principle of the polarographic assay of dissolved oxygen

Oxygen consumption during respiration is recorded by using a **Clark-type oxygen electrode** composed of a reference electrode (silver/silver chloride) and a platinum electrode (negative relative to the reference electrode). Both are immersed in saturated KCl solution. The apparatus is separated from the reaction vessel by polyethylene membrane, which permits dissolved oxygen to enter the electrode surface layer from the reaction mixture, and here, if a voltage is imposed across the two electrodes, oxygen undergoes an electrolytic reduction. When current is plotted as a function of polarizing voltage, first an increase in current (at about 0.4 V), then a plateau region (between 0.5 and 0.8 V) can be observed. The increase in current is caused by the increasing number of oxygen molecules participating in the transport of charges. Then, if voltage is further elevated, the number of oxygen molecules involved in transport reaches a limit determined by their rate of diffusion, which is proportional to the concentration gradient of oxygen established between the bulk of solution and electrode surface ('diffusion-limited current' in the plateau region). By this means, at 0.6 V constant polarization voltage **current becomes directly proportional to the concentration of oxygen**. Current recorded at these conditions is used to calculate changes in oxygen concentration during respiration.

The reaction vessel is kept at a constant temperature and the reaction mixture is stirred continuously during the reaction by the use of a plastic-encased flea driven by a magnetic stirrer to facilitate establishment of the equilibrium between the concentrations of oxygen in the reaction mixture and the electrode space. Reactions are carried out in a closed vessel, so no air bubbles are allowed to be trapped above the medium, and on the inner wall of the vessel. Reactants are carefully pipetted to a hole on the stopper of the vessel, and the force of stirring will drive them inside. After each experiment the vessel is emptied by suction aspiration of the fluid and washed by repeated water rinsing.

Electronic equipment composed of the polarizing voltage source and sensitivity control adjustment are arranged as shown in Fig. 1. Oxygen current is converted to voltage change and recorded by a potentiometric recorder at its highest sensitivity (2 mV/full scale).

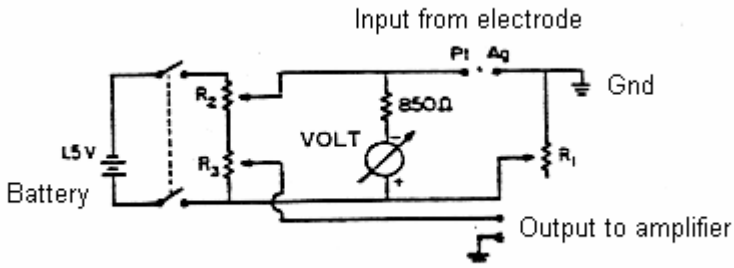

Fig.1. Scheme of the polarizing voltage and sensitivity control of an oxygen electrode. The variable resistor R_1 is sensitivity control, R_2 supplies a polarizing voltage of 0.6 V, R_3 is for zero offset control.

REAGENTS, PREPARATIONS AND EQUIPMENT

Reagents

Incubating Buffer (called 'Medium'): 80 mM KCl, 20 mM Tris, 1 mM EGTA, 10 mM KH_2PO_4, pH 7.2.

Substrates:

0.75 M glutamate with 0.3 M malate, pH 7.2., or

0.75 M succinate, pH 7.2.

Effectors:

50 mM ADP, pH 7.2. To determine an accurate concentration of ADP, dilute 2-thousand-fold an aliquot of this stock and read its absorbance at 260 nm (extinction coefficient = 15.4 $mM^{-1} \times cm^{-1}$)

10 mM 2,4-dinitrophenol (DNP).

0.08 mg/ml oligomycin in abs. ethanol.

0.1 M malonate, pH 7.2.

100mM KCN.

25 mg/ml carboxyatractyloside, pH 7.2.

Na-dithionite solid.

mitochondrial suspension at a protein concentration of 60-80 mg/ml.

Preparation of mitochondria. Sacrifice the rat by cervical dislocation, excise the liver, rinse and place in ice-cold Isolating Buffer. Chop it into small pieces, rinse again, then add 4-fold volume of Isolating Buffer, and homogenize carefully in a Potter-Elvehjem homogenizer (spherical pestle, large clearance), with a few strokes. Repeat homogenization using a tighter pestle of cylindric shape, with a small clearance. Centrifuge homogenate at 600 x g at 0^oC for 8 min, then decant supernatant into a clean centrifuge tube. (Sediment containing largely nuclei and cell debris are discarded.) Centrifuge supernatant at 6-8000 x g for 20 min to pellet mitochondria. Store mitochondria in pellet until use. When needed, suspend the mitochondria carefully in ice-cold Isolating Buffer to about 60-80 mg prot./ml, and keep on ice.

Equipment

Reaction vessel with stopper, teflon-coated stirring flea and magnetic stirrer, attached oxygen electrode with a polarizer unit (needs 1.5 V-battery, polarizer output adjusted to 0.6 V) and a potentiometric recorder.

EXPERIMENTAL PROCEDURE

WARNING! You are working with highly toxic substances. Be very careful!

Use separate tips for each reagent to avoid contamination of stock solutions.

Indicate on your recording when you are adding a reagent. Avoid bubbles remaining in, or getting into the vessel after you start the experiment.

Calibration of the apparatus

The initial saturating concentration of the medium oxygen as a function of temperature is shown in Fig. 2. To use this plot for computing actual oxygen concentration, one needs to know the temperature of the medium. During the calibration procedure a scale between saturated concentration of oxygen and zero oxygen (achieved by dithionite addition) will be established on the potentiometric recorder. To start the calibration procedure turn on the polarizing unit, as well as potentiometric recorder, and pipette 3.5 ml incubation medium in the reaction vessel. Place the stopper (lid) on the the vessel to separate the medium from air, and turn on the stirrer (at the lowest speed), start recorder and pen (chart speed: 60 cm/h (=1 cm/min), at 2 mV/full scale). When the pen is stabilized add a few grains of sodium dithionite close to the hole on the stopper, turn the stopper slightly to permit dithionite to enter the vessel. The pen is now moving toward 0. When it has been stabilized, adjust it to 0 unit (use the zero control knob on the recorder). Zero oxygen concentration has been set. To adjust the recorder pen to saturating oxygen concentration, first remove the stopper, and carefully clean up the vessel and stopper by repeated rinsing with deionized water (use water aspiration, but **take care of the electrode membrane located on the inner wall of vessel at the side arm**). Pipette a fresh medium into the vessel, close the stopper (start stirrer and recorder), and, when the pen is stabilized, adjust its position to 100 unit (use the sensitivity knob on the polarizer unit). After this calibration procedure has been completed, saturating oxygen concentration refers to 100 units on the recorder.

Exp. #1: Determination of respiratory control and ADP/O (P/O) ratio

Add medium (3ml), substrate: glutamate+malate or succinate (20 μl), close the vessel, set the pen at position 100, and wait until no more change occurs in pen position (no oxygen consumption) .
Add mitochondria (50ul) and record the slope of the line on the recorder.
Add ADP (exactly 20 μl of a known stock), and record oxygen uptake rates. After ADP has been utilized and respiration has declined, repeat ADP addition, and record respiration again. Use both sets of ADP addition to calculate P/O ratio (see attached sheet).

Exp. #2: Effects of ADP, oligomycin, DNP and KCN on respiration

The experiment may be carried out using the substrate succinate, or glutamate+malate. Proposed procedure:

add medium (3.0 ml) + substrate (20 μl), close the vessel with stopper, and wait until no more change occurs in pen position (roughly 1 min);

add mitochondria (50 μl) to the hole on the stopper and when respiration stabilizes add ADP (50 ul) and after recording the accelerated respiration,

add oligomycin (20 μl), wait and establish the low rate of the oxygen consumption;

add DNP (20 μl) to uncouple respiration from phosphorylation, check if DNP has really restored fast oxygen consumption;

inhibit respiration by the addition of KCN (20 μl); if the oxygen uptake is ceased,

add a few grains of dithionite to demonstrate that oxygen is still present in the medium.

Exp. #3: Effects of carboxyatractyloside on respiration

The experiment may be carried out using the substrate succinate, or glutamate-malate. Proposed procedure:

add medium (3.0 ml) + substrate (20 μl), close the vessel with stopper, and wait until no more change occurs in pen position (roughly 1 min);

add mitochondria (50 μl) to the hole on the stopper and when respiration stabilizes add ADP (50 ul) After recording the accelerated respiration,

add carboxyatractyloside (20 μl), and establish the diminished rate of the oxygen consumption;

add DNP (20 μl) to uncouple respiration from phosphorylation, check if DNP has really restored fast oxygen consumption;

Inhibit respiration by the addition of KCN (20 μl); if the oxygen uptake is ceased,

Add a few grains of dithionite to demonstrate that oxygen is still present in the medium.

Exp. #4: Inhibition of succinate oxidation by malonate

add medium (3.0 ml) + succinate (20 μl), close the vessel with stopper, and wait until no more change occurs in pen position (roughly 1 min);

Add mitochondria (50 μl) to the hole on the stopper and when respiration stabilizes add ADP (50 μl). Record the accelerated rate of respiration for about a minute, then add malonate (20 μl). When oxygen uptake slowed steadily down, add succinate (100 μl) to cease inhibition.

EVALUATION OF RESULTS

1. Calculate the rates of *'resting'* and *'active'* respiration in μmole O_2/min/μl for the respiratory substrates used.
2. Calculate the RC values for the two substrates.
3. Calculate P/O (ADP/O) for the substrate used.
4. Evaluate the effects of respiratory inhibitors and uncoupler.

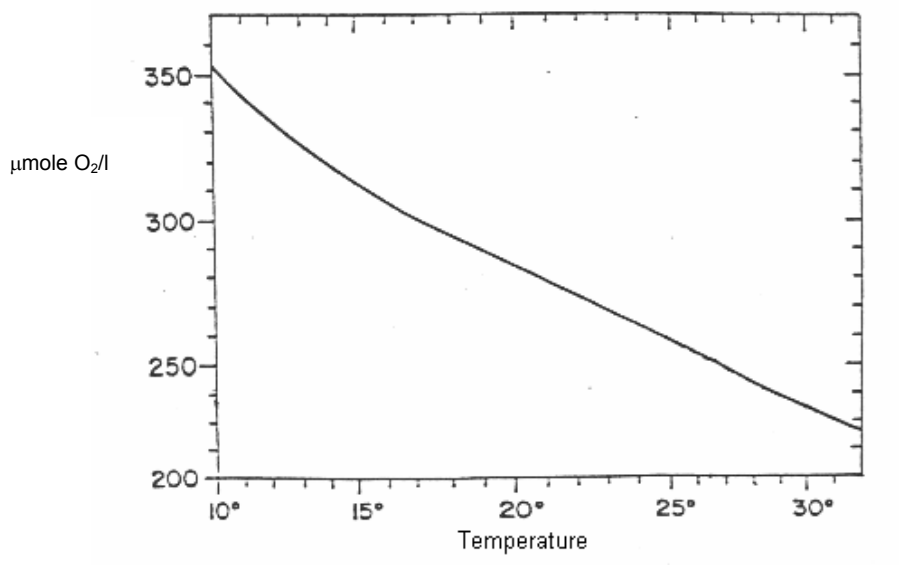

Fig.2. Oxygen content of air-saturated water versus temperature.

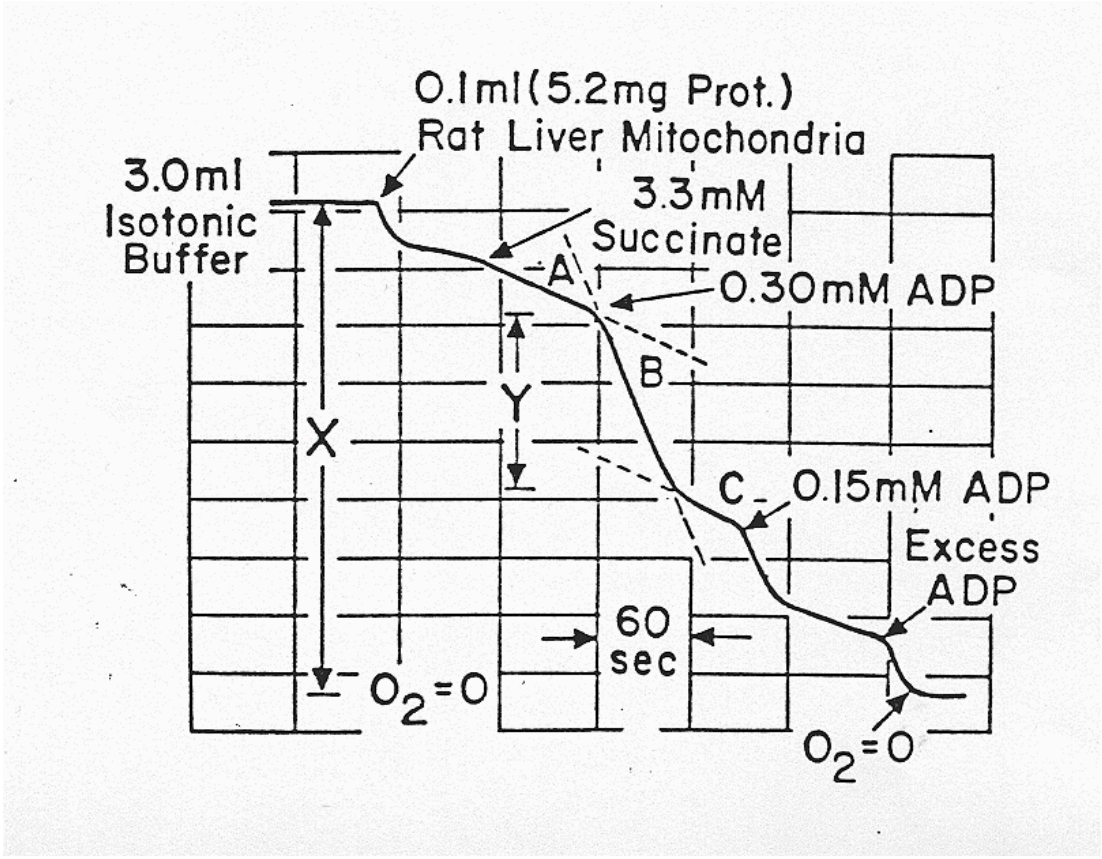

Fig.3. Determination of ADP/O (P/O) ratio from experimental data

CALCULATION OF ADP/O (P/O) RATIO

(See Fig. 2. and 3. for details)

Final volume:		ml
Temperature:		^{o}C.
Saturating conc. of O_2 at this temp.:		μmole/ml.
Total oxygen content ('X'):		μmole
Oxygen used for ADP ('Y'): .....units =		μmole
Substrate for the respiration:		
Stock solution used:		mM
Volume added		μl
ADP stock:		mM
Volume added:		ml
Amount of ADP used:		μmole

$$\text{ADP / O (P/O) ratio} = \frac{\text{ADP}, \mu\text{mole}}{2 * \text{O}_2, \mu\text{mole}} = \frac{.....}{2 * .....} = ...$$