Oxidative Phosphorylation

Introduction

- **Oxidative phosphorylation** is the process by which **ATP** is formed as a result of the **transfer** of **electrons** from **NADH** or **$FADH_2$** to **O_2** by electron carriers.
- The energy in **NADH** and **$FADH_2$** is used to create a **proton gradient** across the membrane. This gradient is then used to form **ATP** from **ADP**. Thus, **proton gradients** are an **interconvertible currency of free energy** in biological systems.
- **Oxidative phosphorylation** occurs in the **inner membrane** of the **mitochondria**, which has a rather large surface area thanks to **cristae**, which are **infoldings** of the **inner membrane**. The space **inside this membrane** is the **matrix**.
- The **outer membrane** of mitochondria is fairly permeable to most species [due to **porins** embedded therein]. The **inner membrane**, however, is impermeable to most things. We refer to the inner face of the inner membrane as the **matrix side**, and the outer face as the **cytoplasmic side**.
- The overall reaction involves **too large** a change in energy to occur in **one go**. The **electron transport chain** allows it to occur in **small stages**, each of which do not **waste** so much energy.
- Note that the **stoichometry** of the **different carriers** in the membrane is *not* 1:1, and there is no real "chain" as such. They each act **independently**.

Redox Potentials

- The **redox potential** of a substance is its **affinity for electrons**, and is measured in **volts**, relative to the affinity of H_2 to electrons. A **negative** redox potential indicates **less** affinity to electrons than H_2.

- This means that the **transfer of electrons** from a species with **low** redox potential to one with **high** redox potential will be **exothermic**. In fact, for such a reaction:

$$\Delta G^{\circ'} = -nF\Delta E'$$

  Where n is the number of electrons involved in the transfer.
- They are measured by comparison with an $H^+{:}H_2$ half-cell, which is by definition given a redox potential of 0. Biochemists use a hydrogen electron with an H^+ concentration that gives a pH of 7 (denoted E'_0).
- Redox potentials at other concentrations of oxidant and reactant can be worked out using

$$E' = E'_0 + \frac{RT}{F\log_{10} e}\log_{10}\left(\frac{[\text{Oxidised carrier}]}{[\text{Reduced Carrier}]}\right)$$

- By convention, **redox potentials** refer to partial reactions written as **Oxidant + *n*e$^-$ → Reductant**.
- The **driving force** of **oxidative phosphorylation** is the **electron-transfer potential** of **NADH** or **$FADH_2$** relative to that of **O_2**. The pertinent half-reactions are:
  - ½ O_2 + 2 H^+ + 2 e^- → H_2O $\left(E'_0 = +0.82V\right)$
  - NAD^+ + 2 H^+ + 2 e^- → NADH + H^+ $\left(E'_0 = -0.32V\right)$
  - FAD + 2 H^+ + 2 e^- → $FADH_2$ $\left(E'_0 = -0.22V\right)$
- The energy released is used to pump protons across the membrane. We know that the free-energy change for a species moving from one side of the membrane (where it is a concentration c_1) to the other (where it is at concentration c_2) is

$$\Delta G = \frac{RT}{\log_{10} e}\log_{10}\left(\frac{c_2}{c_1}\right) + ZF\Delta V$$

  Where Z is the charge of the species and ΔV is the membrane potential.

The electron Transport Chain

Electrons are transferred **from NADH** to **O_2** through a chain of three large protein complexes:

- **Complex I** – NADH-Q oxidoreductase (also called NADH dehydrogenase).

  This is an enormous enzyme consisting of about 46 polypeptide chains. It is L-shaped, with one arm lying **in the membrane**, and the other **projecting into the matrix**. The reaction catalysed by the enzyme is

  $$\mathrm{NADH} + \mathrm{Q} + 5\mathrm{H}^+_{\mathrm{matrix}} \rightarrow \mathrm{NAD}^+ + \mathrm{QH}_2 + 4\mathrm{H}^+_{\mathrm{cytoplasmic}}$$

  The steps of the reaction are as follows:

  - ***In the extramembranous portion*** – **NADH binds** and transfers its **high-potential electrons** to the ***flavin mononucleotide*** **(FMN)** prosthetic group of the complex, to give the reduced form **$\mathbf{FMNH_2}$**.
  - ***In the extramembranous portion*** – These electrons are then transferred through a number of **iron-sulphur clusters**, the second type of prosthetic group, of which there are more than five. Complex I contains **2Fe-2S** clusters as well as **4Fe-4S** clusters (see Stryer p. 511). The irons cycle between Fe^{2+} and Fe^{3+} without the need for protons.
  - ***In the intramembranous portion*** – the electrons are finally passed to **Q**, which as a consequence accepts two protons from the **matrix**. It is then released into the **hydrophobic interior** of the membrane.

- **Complex II** – Succinate-Q reductase (which contains the enzyme **sucinate dehydrogenase** which generates $FADH_2$ in the citric acid cycle).

  The electrons in $FADH_2$ are **transferred** to **Fe-S centres** in the complex, and then directly to Q. No protons are transported, and so less ATP is generated from $FADH_2$.

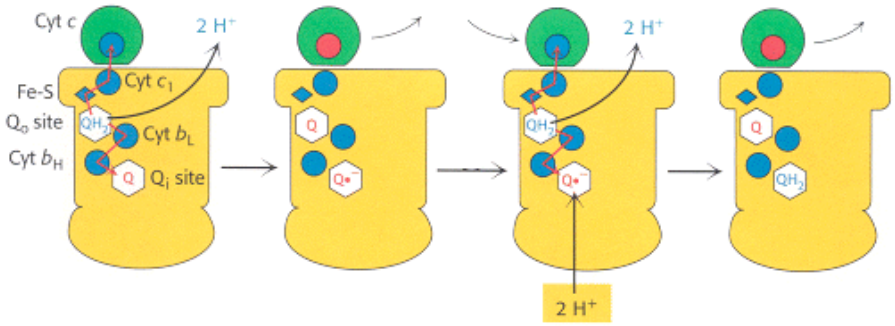
- **Complex III** – Q-cytochrome *c* oxidoreductase

  The function of this complex is to catalyse the transfer of electrons from **$\mathbf{QH_2}$** to oxidised **cytochrome *c***, and to concomitantly pump protons out of the matrix. The reaction is:

$$QH_2 + 2\ \text{Cyt}\ c_{ox} + 2\ H^+_{matrix} \longrightarrow Q + 2\ \text{Cyt}\ c_{red} + +2\ H^+_{cytoplasmic}$$

(Note that less protons are pumped because of the smaller thermodynamic pumping force).

This enzyme contains **two cytochrome *b*** and **one cytochrome c_1** (and it is sometimes called the **bc_1 complex**). The protein also contains a 2Fe-2S centre. The mechanism of this enzyme is **complex**, because it must **funnel electrons** from a **2-electron carrier** to a **1-electron carrier**:



Notes:

1) In sum **four protons are released on the cytoplasmic side** and **two electrons are removed from the matrix side**. Two **QH_2** are converted to **Q**, and **one Q** is converted to **QH_2**.
2) The **Cytochrome *b*** mechanism in effect just "recycles" one electron.

- **Complex IV** – Cytochrome *c* oxidase

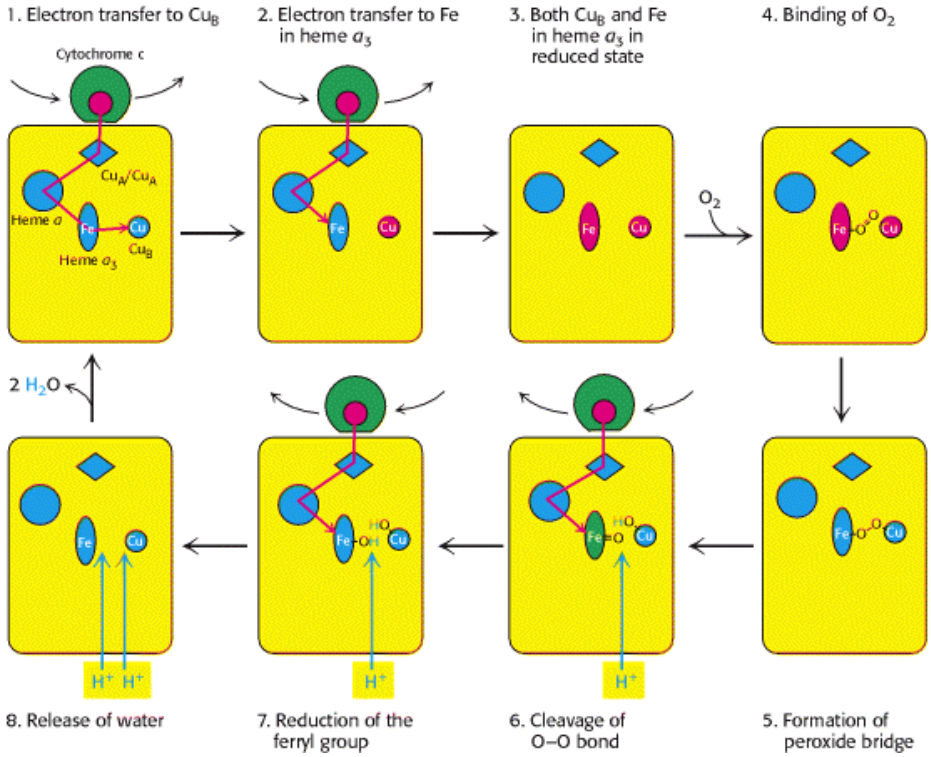
  This complex transfers electrons from **reduced Cytochrome *c*** to the **final electron acceptor**, **O_2**. This, again, increases the proton gradient:

$$4\ \text{Cyt}\ c_{red} + 8\ H^+_{matrix} + O_2 \longrightarrow 4\ \text{Cyt}\ c_{ox} + 2\ H_2O + 4\ H^+_{cytoplasmic}$$

  This enzyme contains:

  - **Two copper centres** designated **A** and **B** – the first, ***Cu_A/Cu_A***, contains **two copper ions** linked by **two bridging cysteine residues**. These alternate between Cu^+ (**cuprous**) and Cu^{2+} (**cupric**) as they accept and donate electrons.
  - Two **heme A molecules**, called **heme *a*** and **heme a_3**. These have **distinct redox potentials** because they are located in **different environments**.

The mechanism is as follows:



Notes:

1) In steps 6 and 7, it is the addition of more electrons that reduce the two complexes bound to oxygen.
2) The electrons for the reaction come **exclusively** from the **cytoplasm**, and so they contribute to the proton gradient.
3) As well as the mechanism above, the protein pumps an **additional 4 protons** from the **matrix** into the **cytoplasm**. The mechanism by which this occurs is badly understood, but we have a few clues:
    - **Charge neutrality** tends to be maintained in the interior of proteins. Thus, binding of an electron at one site encourages the binding of a proton at another.
    - Some **conformational change** seems to occur around the a_3-Cu_B centre in the course of the reaction.
4) If the oxygen is not **fully reduced**, then **toxic by-products** can be formed. This is prevented by the enzyme keeping a **tight grip** on the oxygen (between an **Fe** and a **Cu** atom) and through a **variety** of **other enzymes** that **scavenge for** and **destroy** the other ions.

Complexes I, II and III appear to be associated in a **supramolecular complex** termed a **respirasome**. These facilitate the **rapid transfer** of substrate and prevent the release of **intermediates**.

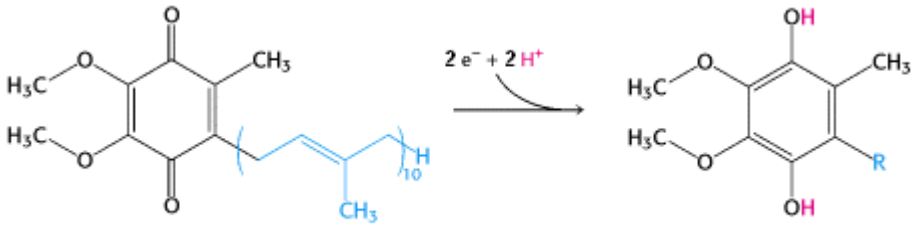
Electron flow within the transmembrane complexes **I**, **III** and **IV** leads to the **transport of protons** across the **inner mitochondrial membrane**. Complex **II** does not pump electrons, and simply *inserts* electrons from **$FADH_2$** into the transport chain (into Complex III).

Special **electrons carriers** carry electrons from one complex to the next:

- **Complex I → Complex III** is handled by the **reduced form** of ***coenzyme Q***, also known as **ubiquinone**.

  This is a **hydrophobic quinone**, that diffuses **rapidly** within the **inner mitochondrial membrane**.

  Unbiquinone is a **quinone derivative** consisting of a number of **five-carbon isoprene units**. The most common form in mammals contains **10** of these units.



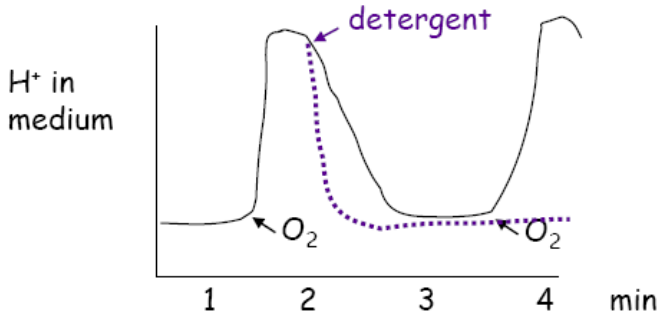
  The reduction of Q to QH_2 proceeds through a **semiquinone intermediate** (QH•) in which only **one** of the keto groups is **reduced**. E**electron transfer** in Q is **coupled** to **proton binding** and **release**.
- **Complex II → Complex III** is also handled by **Q** (**ubiquinone**).
- **Complex III → Complex IV** is handled by **Cytochrome *c***, a **small**, **soluble protein**.

  A **cytochrome** is an **electron-transferring protein** that contains a **heme prosthetic group**. The iron ion of a cytochrome **alternates** between a **reduced ferrous** (+2) and **oxidised ferric** (+3) state.

Chemiosmosis

Chemiosmosis was proposed by **Pater Mitchell**, and won him the nobel prize. Originally, people leaned towards a "high energy intermediate" hypothesis, in which ATP was eventually formed by substrate-level phosphorylation. There is lots of evidence that this is the correct theory:

- ***pH gradients are, indeed, observed as predicted*** – There is a **rapid change in the pH of the external medium** when **electron transfer** is **stimulated** by the introduction of $\mathbf{O_2}$ (mitochondria) or **light** (chloroplasts).

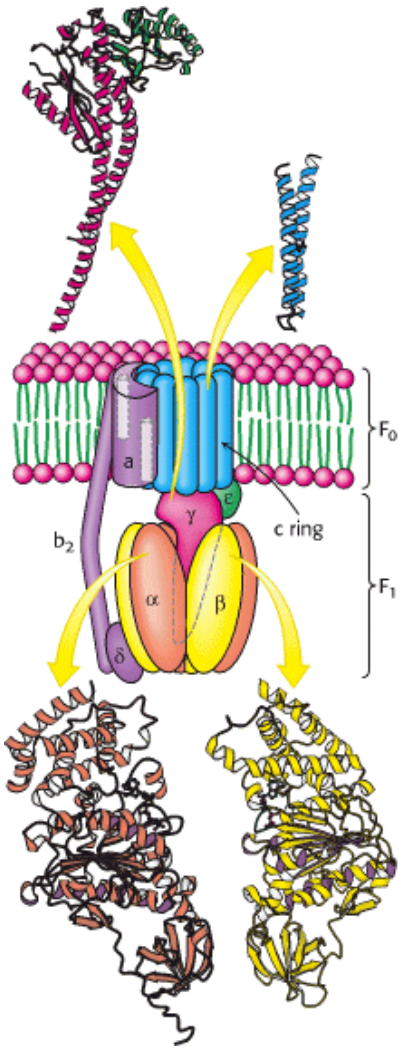


  If a **detergent** or **uncoupler** is added, this effect **stops**.
- The action of **uncouplers**, such as **DNP** or **FCCP**, **stop** these **pulses** [see later].
- **Membrane voltages** are, indeed, observed.
- An experiment was done by **André Jagendorf** in **1966**, in which he **soaked thylakoids** in a **pH 4 buffer** for several hours, and then **rapidly resuspended them with a pH 8 buffer containing ADP** and $\mathbf{P_i}$. A **sharp burst** of **ATP synthesis** accompanied the **dissipation of the proton gradient**. Addition of **FCCP prevents** the formation of **ATP**.

Under typical conditions, the **p.m.f.** in a cell is about **21.8 kJ** per mole of protons.

ATPase is a **stalked particle**, which appears clearly on microscope pictures. The "**stick**" part (the $\mathbf{F_0}$ **domain**) is **embedded into the inner mitochondrial membrane**. It contains:

- The **proton channel** of the complex, which consists of a **ring** of 10-14 **carbon subunits** that are **embedded** in the membrane.
- A single **a** subunit binds to the outside of the ring.
- Two **b** subunits connect the **a** subunit to the δ subunit of the "ball", connected to the **outside** of the $\alpha_3\beta_3$ hexamer.



The "**ball**" part (the $\mathbf{F_1}$ **domain**) **protrudes into the matrix**. The $\mathbf{F_1}$ **unit** contains the **catalytic activity** for the synthase. It contains:

- Three α and three β subunits, arranged **alternatively** in a **hexameric ring**.
- A **central stalk** consisting of a γ and an ε protein. The γ protein has a long **helical coil** that extends **into** the $\alpha_3\beta_3$ hexamer. This **breaks the symmetry** of the hexamer: each of the units is **distinct** by virtue of its **interaction** with a **different face** of γ.

We can prove that the $\mathbf{F_1}$ domain synthesises ATP whereas the $\mathbf{F_0}$ domain allows the passage of protons:

- **Isolate the inner membrane** of a **mitochondrion** and **sonicate it**. This forms **small vesicles**.
- Treat with **salt**, to remove the $\mathbf{F_1}$ part.
- The membranes left behind are **leaky to protons**, and the $\mathbf{F_1}$ part can now **hydrolyse ATP**.

ATPase catalyses the formation of **ATP** from **ADP** and **orthophosphate**:

$$ADP^{3-} + HPO_4^{2-} + H^+ \longrightarrow ATP^{4-} + H_2O$$

In **reality**, the substrates actually attack as $\mathbf{Mg^{2+}}$ **complexes**. The mechanism involves a **terminal oxygen atom** in the **protonated orthophosphate attacking the ADP**, and the resulting molecule **dissociating** into ATP and H_2O.

It turns out, however, that the **proton flow** is **not** needed to synthesise ATP. Studies involving radioactive $\mathbf{H_2{}^{18}O}$ in the **absence** of a **p.m.f.** revealed that **ATP** was nevertheless formed from **already-bound ADP**. However, it **did not leave the active site**.

A mechanism [Paul Boyer] for **ATPase** is as follows:

- Depending on which face of γ each of the β subunits are bound to, they can take one of **three** conformation [ie: binding with different faces of the γ subunit causes **conformational changes** in β]:
  - The **Lose (L)** conformation, which **binds ADP and P_i**.
  - The **Tight (T)** conformation, which **binds ATP so strongly** that it will convert **ADP** and P_i into **ATP**.
  - The **Open (O)** conformation, which binds a nucleotide, but can also **release** a **bound nucleotide**.
- Each **turn** of the γ subunit causes the **state** of the proteins to **cyclically rotate**. [Rotation is actually **anticlockwise**].
- The rotation is driven by the flow of protons through the F_0 domain of ATPase, like so [Berg & Oster]:
  - The **a** subunit is believed to contain two **hydrophilic channels**, spanning just over half the protein, one with an opening to the **matrix** side, and one with an opening to the **cytoplasmic** side.
  - The **c** subunits, however, contain *two* helices linked by an **aspartic acid** in the middle.

  1) A proton enters into the **cytoplasmic channel** of the **a** subunit. It is transferred to the **aspartic acid**, which becomes **neutral**.
  2) The **neutralised aspartic acid** now has the **ability** to occupy the **hydrophobic environment of the membrane** (outside the **a** subunit), and wants to **leave** the **hydrophilic environment** of the channel. It therefore **rotates**.
  3) A new **c subunit** therefore comes close to the **matrix channel** of the **a subunit**. It **loses its proton** through the channel **into the matrix**, and becomes **charged**.

4) The **c subunit** that was **previously** near the **matrix channel** and therefore **charged** *moves* to be next to the **cytoplasmic channel**, and step (1) occurs again.

- The **c ring** is **tightly bound** to the γ and ε subunits. The **number of c subunits in the ring** is **important**, because it dictates the **number of protons needed** per molecule of **ATP**.

- The **exterior column** consisting of the two **b** chains and the δ subunit prevents the tetramer from rotating.

For this to be able to occur **ADP** must be readily available in the mitochondrion.

- The **entry of ADP** is coupled to the **exit** of **ATP** by **ATP-ADP translocase**, a **specific transport protein** which makes up about 15% of the protein in the **inner membrane** of the mitochondrion.
- The protein contains a **nucleotide binding site** that **alternately** faces the **cytoplasmic** and **matrix** side of the membrane.
- **ADP** has **3 negative charges** and **ATP** has **4 negative charges**. Thus, in an actively respiring mitochondrion with a **positive membrane potential**, this process is **favourable** but **very expensive**, since it **dissipates** the proton gradient.
- This is an example of **another use organisms** make of **proton gradients** (apart from making **ATP**). In **bacteria**, such gradients are similarly used for transport, and sometimes *directly*, to produce **flagellar rotation**. [Note that bacterial electron transport chains use other electron acceptors as well as O_2].

Regulation of Cellular Respiration

The **ATP needs of the cell** are the **ultimate determinant** of the r**ate of respiratory pathways**. It turns out that **30 molecules of ATP** are generated by the **complete oxidation of glucose**. These come from:

- **2 ATP** from **anaerobic glycolysis**.
  - **2 $NADH_{cytoplasm}$** are produced.

- The conversion of **pyruvate** into **Acetyl CoA** generates no **ATP**, but
  - **1 × 2 = 2 NADH** are produced.
- **1× 2 = 2 ATP** from the **citric acid cycle**.
  - **3 × 2 = 6 NADH** are produced.
  - **1 × 2 = 2 $FADH_2$** are produced
- Finally, **oxidative phosphorylation**. Assuming **10 c units** in **ATP synthase** (as was observed in yest), each **ATP** requires the passage of **10 ÷ 3 ~ 3 protons** through **ATP synthase** and an **additional proton** is **consumed** in transporting **ADP** into the matrix. Thus:
  - The **2 $FADH_2$** produce **1.5× 2 = 3 ATP**.
  - The **2 $NADH_{cytoplasm}$** produce as much energy as **$FADH_2$ = 3 ATP**.
  - The **8 NADH** produce **2.5 × 8 = 20 ATP**

The entire process is regulated by the **phosphorylation of ADP to ATP**. When this is not needed (and therefore **ADP** is not present), the entire process [the citric acid cycle and the electron transport chain] is slowed down simply because the materials needed by these process are **not available**:

- The **proton gradient** is **not dissipated** by the production of **ATP**. Therefore, electrons are **not transferred** from **$FADH_2$** and **NADH** to **O_2**.
- As a result, the concentration of **$FADH_2$** and **NADH** is high, and the concentration of **FAD** and **NAD^+** is low – the **citric acid cycle** therefore **slows down**.

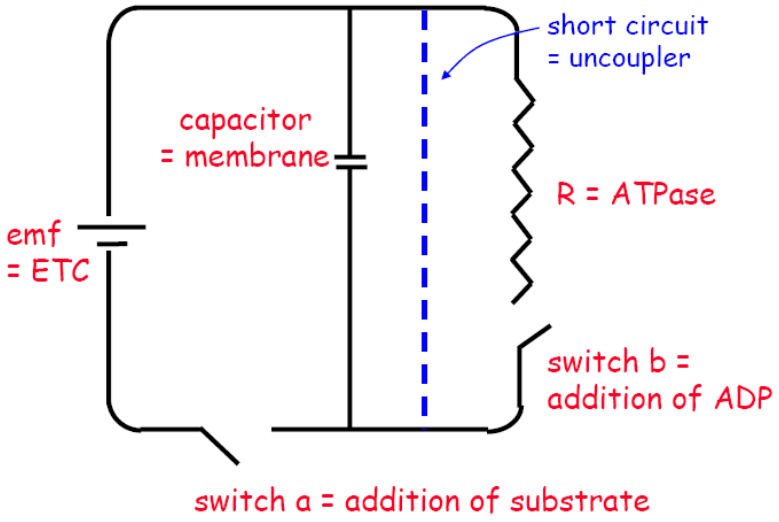
This can be seen very clearly in experiments tracking the **oxygen concentration** in a liquid containing **mitochondria**. The O_2 **consumption increases** when **ADP** is added, and eventually slows down, when the **supply of ADP is exhausted**.

Some organisms, however, posses the **ability to uncouple oxidative phosphorylation** form **ATP synthesis**, and use this ability to **generate heat**. Such organisms have an **uncoupling protein** (**UCP-1** or **thermogenin**) in their **inner mitochondrial membrane**, that allows the **influx of protons into the matrix *without* the synthesis of ATP**. This releases the energy usually

captured in ATP as **heat**. In effect, uncouplers **short-circuit the mitochondrial proton battery**. Examples of where this occurs:

- In some **newborn animals** (including humans).
- Mammals adapted to the cold. In **animals**, **brown fat** is **specialised tissue** for this process, called **nonshivering thermogensis**. The fat looks **brown** because of a combination of the **greenish cytochromes** in the **many mitochondria**, and the **red blood vessels** which are abundant there.
- The **skunk cabbage**, to heat its **floral spikes** in **early spring**, increasing the **evaporation** of **odoriferous molecules** that attract insects.

In a way, the whole process can be compared to an **electrical circuit**:



Experimental Methods

The process of electron transfer can be tracked through **difference spectra** – the **absorbance** is measured under **reduced** and **oxidised** conditions, and the results are display as **one subtracted from the other**. If the result is **non-zero**, this indicates that **redox components** are present. The different components have **distinct peaks**.

Fe.S centres and **Cu** centres can be monited using **electron paramagnetic resonance** (EPR) – it turns out that **unpaired electrons absorb microwaves**. The **height** of the peak reveals the **extent** of the reduction.

The **components** of the **mitochondrion** can be investigated as follows:

- **Mild treatment** releases **extrinsic proteins** (eg: ATP-F_1, cytochrome *c*)
- The membrane can then be **solubilised** with **detergent**, and the **protein complexes** are released.

The detergent can then be **suddenly removed** and **replaced with phospholipids**. **Liposomes** are then formed, which can be used to **investigate the action** of the **protein complexes**.

Bacteriorhodopsin can also be **embedded** into those **synthetic vesicles**. It plays the role of the **electron transport chain** by **pumping electrons out** of the **vesicle** when **illuminated**.

Redox potentials suggested the order of the chain as laid out above. **Specific inhibitors** provided the evidence. We can **block** the chain at a given point, and find that there is a **build up of electrons** *before* the block, and a **deficit of electrons** *after* the block. These are detected by:

- Changes in **absorbance**.
- Changes in the **amount of O_2 consumed**.

Specific inhibitors are:

- **Complex I** (**NADH dehydrogenase**) is **blocked** by **rotenone** [a fish and insect poison] and **amytal** [a barbiturate sedative]
- **Complex III** (cyt bc_1 **complex**) is **blocked** by **antimycin A**.
- **Complex IV** (**cytochrome oxidase**) is **blocked** by **CN^-** and **azide** (**N_3^-**) **ions**, as well as **carbon monoxide**. The former react with the **ferric form** of **heme a_3** and the latter inhibit the **ferrous form**.
- **FAD-linked dehydrogenases** (eg: succinate dehydrogenase in Complex II) can produce **ubiquinone** *without* the need for anything from Complex I if fed the appropriate substrate (eg: **succinate**, the intermediate of the **citric acid cycle**).
- **Ascorbate** can **by-pass** any between **Complex III** and **Complex IV** by **directly reducing cytochrome *c***.

- **Oligomycin** (an antibiotic used as an antifungal agent) and **DCCD prevent** the influx of protons through **ATP synthase**. [The fact that the electron transport chain stops when this is added (ie: O_2 uptake is inhibited) provides a clear indication that the electron transport chain and ATP synthesis are tightly coupled].
- **Chemical uncouplers** are **unregulated counterparts** of **coupling protein**. For example, **2,4-dinitrophenol (DNP)** and certain other acidic aromatic compounds allow the flow of protons across the mitochondrial membrane. When these are consumed, the proton gradient is dissipated without the formation of ATP, and large amounts of fuels are consumed and the energy released as heat. DNP:
    - Is the **active ingredient** of some herbicides and fungicides.
    - Was **banned** as a weight-loss drug by the FDA in 1938.
    - Is rumoured to have been fed to Soviet soldiers to keep them hot during the long Russian winters.
- The action of ATP-ADP translocase can be inhibited by very low concentrations of **atractyloside** (binds when the active site faces the cytoplasmic side) or **bongkrekic acid** (binds when the active site faces the matrix site). Oxidative phosphorilation stops soon after this is added, proving once again what we have discussed.
- Similarly, in **photosynthesis**, **DCMU** can **block electron transfer** to **PQ** in **photosystem II**. This interrupts the **photosynthetic electron transport chain**.

The **action** of **ATPase** can be observed using a **cloned version** of the enzyme containing only $\alpha_3\beta_3\gamma$ units. The β subunits were tagged with **polyhistidine**, with a **high affinity** for **nickel ions**, and could be **bound** to a **glass surface covered** with **nickel ions**. The γ subunit was linked to a **fluorescent actin filament** that could be observed under a **microscope**. It was found that **addition of ATP** caused the actin filament to **rotate unidirectionally** (**anticlockwise**) in 120° increments.

Evolution of Electron Transfer Chains

Electron transport chains are supposed to have evolved as follows:

- Originally, **fermentation** and **substrate level phosphorylation** were used to produce **ATP**. End products (usually **acids**) were **excreted** and **increased** the acidity of the environment.
- **H^+-ATPases** evolved, to **prevent** the **acidification** of cells (*used* ATP).
- **H^+** pumps evolved, as part of **primitive ETCs**. They used **inorganic ions** or **organic acids** as **acceptors**.
- H^+ **gradients** could be used to **drive H^+-ATPasaes backwards**, to **make ATP**.
- **Photosynthesis evolved** to use **light**. H_2S was the original electron acceptor, followed by H_2O.
- **Rising** levels of **O_2** in the **atmosphere** led to **ETCs** that could use it as a **final acceptor**.