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Inhibitors of Mitochondrial Function

Editors:
MARIA ERECIŃSKA
and
DAVID F. WILSON

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PREFACE

THIS section of the *International Encyclopedia of Pharmacology and Therapeutics* deals with inhibitors of mitochondrial functions. Since the elegant work of Webb (1966), which was unfortunately never completed, no major effort has been made to summarize concisely the information which has accumulated over the years. The present book is intended to fulfill such a need. It covers a variety of topics ranging from inhibitors of the many mitochondrial enzymes to agents which interfere with movements of ions across mitochondrial membrane. The emphasis of this volume has deliberately been focused on "hard facts" rather than scientific theories. It is our feeling that experimental results have timeless value whereas working hypotheses, even the most elegant ones, often enjoy a rather short-lived period of recognition. However, in order to present a comprehensive picture of the various topics, each contributor has been asked to provide her/his chapter with a short introductory section and an extensive list of references. Moreover, the book has been supplemented with a general introduction which is intended as a guide for those who are not already directly involved in studying mitochondrial metabolism.

We hope that *Inhibitors of Mitochondrial Functions* will serve as an effective source of reference material and a useful investigational aid. As in any venture of this kind the reader is the ultimate authority on its failure or success. Consequently, any suggestions that you may have concerning either the content or organization of this book will be most appreciated and will assist us in preparing future editions of this volume.

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CHAPTER 1

THE MITOCHONDRION AND ITS FUNCTIONS

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Members of the animal kingdom, in common with other nonphotosynthetic organisms, lack the biochemical apparatus required to utilize light as a source of energy to drive essential endergonic reactions. For this reason all nonphotosynthetic organisms must rely on an external supply of foodstuffs that can be utilized, primarily through reduction of molecular oxygen, as a source of metabolic energy. The most common form in which the energy liberated during combustion of foodstuffs is stored and transmitted to sites of utilization is the pyrophosphate bond of adenosine triphosphate (ATP). The pyrophosphate bonds are often referred to as 'high-energy' bonds because the free energy of change associated with their hydrolysis is more negative than approximately -6 kcal/mole. This value is much higher than that of usual ester-hydrolysis reactions (-2 to -4 kcal/mole).

The release of energy by the combustion of foodstuffs in living matter may be regarded as occurring primarily in three major phases. In phase I large molecules of food: glycogen, lipid, protein and nucleic acids are broken down to their smaller constituent units (sugars, fatty acids, amino acids, etc.). The amount of energy liberated in phase one is rather small (below 1%). In phase II, the various small molecules, sugars, amino acids, fatty acids, are partially oxidized to CO_2, H_2O and one or more of three substances, acetyl-CoA, α-ketoglutarate, and oxaloacetate, which are metabolically closely interrelated. In mammalian cells they are then completely oxidized to CO_2 and H_2O by the tricarboxylic acid cycle (phase III) which is coupled to oxidative phosphorylation. Approximately 2/3 of the total energy of combustion is liberated in this final phase, the other 1/3 being set free in phase II.

The enzymic systems responsible for the terminal oxidation of foodstuffs, the tricarboxylic acid cycle and oxidative phosphorylation (phase II), are enclosed in a separate cellular compartment, an organelle called the mitochondrion. It is because of its key role in cellular bioenergetics that the mitochondrion has been termed the power-house of the cell.

Mitochondrial Structure

The shape and the number of mitochondria per cell differ widely from cell to cell (for detailed review see Lehninger's *The Mitochondrion* and Wainio's *The Mammalian Mitochondrial Respiratory Chain*). In the liver and muscle cell, the mitochondria are approximate ellipsoids, about 2–3 μ long and about 1 μ thick. In liver these organelles constitute about 10–15% of total cell volume, in cultured mouse neuroblastoma only 5%, and in the heart perhaps as much as 20%. The mitochondrion has two membranes: the inner and outer one. The inner membrane, which is highly invaginated, forms the so-called cristae and encloses a space termed the mitochondrial matrix. The outer membrane is relatively smooth and resembles the endoplasmic reticulum in structure and enzymatic composition. The space between the inner and the outer membranes is called the intermembrane space.

The main building-blocks of the mitochondrion are proteins and lipids with a small amount of carbohydrate found mainly on the cytoplasmic surface of the outer membrane. The primary function of the lipids is to form a permeability barrier, whereas the

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TABLE 1. *Localization of Mitochondrial Enzymes*

Outer Membrane	Inner Membrane	Intermembrane Space	Matrix
Acyl-CoA synthetase, enzymes of phospholipid metabolism (glycerophosphate acyltransferase, lysophosphatidate acyltransferase, phospholipase A_2), kynurenine 3-monooxygenase, amine oxidase, cytochrome b_5-reductase.	Succinate dehydrogenase, enzymes of the respiratory chain [NADH dehydrogenase, ubiquinone-cytochrome *c* oxidoreductase (cytochrome bc_1 complex), cytochrome oxidase, adenosine triphosphatase] enzymes structurally and/or functionally-linked to respiratory chain (choline dehydrogenase, α-glycerophosphate dehydrogenase, proline oxidase, $NAD(P)^+$ transhydrogenase, enzymes of steroid metabolism, ferrochelatase, anion transport systems, cation transport systems.	Adenylate kinase, creatine kinase, nucleoside-diphosphate kinase, sulfite oxidase, d-xylulose reductase.	Enzymes of the tricarboxylic acid cycle [citrate synthase, aconitase, isocitrate dehydrogenases (NAD^+ and $NADP^+$-dependent), α-ketoglutarate dehydrogenase, lipoamide dehydrogenase, succinyl-CoA synthetase, fumarate hydratase, malate dehydrogenase], enzymes ancillary to TCA cycle [glutamate dehydrogenase (NAD^+(P)-dependent), methylmalonyl-CoA mutase, methylmalonyl-CoA racemase, phosphoenol pyruvate carboxykinase (GTP), propionyl-CoA carboxylase, pyruvate carboxylase, pyruvate dehydrogenase], enzymes activating fatty acids [acetyl-CoA synthetase, butyryl-CoA synthetase, acyl-CoA synthetase (GTP)], acyl carnitine transferases (acetyl and palmitoyl transferases), enzymes of fatty acid oxidation (butyryl-CoA dehydrogenase, acyl-CoA dehydrogenase, electron-transferring flavoprotein, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, acetyl-CoA acyltransferase, Δ^3-**cis**-Δ^2-**trans**^{1}-enoyl-CoA isomerase, 3-hydroxybutyryl-CoA epimerase) fatty acid elongation system, enzymes of ketone body metabolism (acetyl-CoA acetyltransferase, hydroxymethyl-glutaryl-CoA lyase, 3-hydroxy-butyrate dehydrogenase, 3-ketoacid-CoA transferase), enzymes of nucleic acid and protein metabolism, aminotransferases, nucleoside diphosphate kinase, enzymes of heme biosynthesis (δ-aminolevulinate synthase, coproporphyrinogen oxidase), enzymes of urea cycle (carbamoyl-phosphate synthase, ornithine carbamoyl-transferase), aldehyde-dehydrogenase, dimethylglycine dehydrogenase, 1-pyrroline dehydrogenase, sarcosine dehydrogenase, 1-xylulose reductase.

The information was taken from 'Cell Biology', Biological Handbook series. P. L. Altman and D. D. Katz (Eds.) FASEB, Bethesda, MD 1976. Some enzymes may exhibit dual locations or may become loosely attached to one of the membranes during isolation procedure. Therefore for details the reader should consult the original references given in 'Cell Biology'.

proteins perform the multiple catalytic activities of the organelle. The various enzymes are either tightly bound to one of the mitochondrial membranes or are soluble in one of the aqueous spaces created by the double membrane structure. For the convenience of the reader, a list of some mitochondrial enzymes and their various locations has been compiled in Table 1.

The Inner Mitochondrial Membrane and the Matrix Space

The enzymes of the tricarboxylic acid cycle, fatty acid oxidation and of other metabolic pathways (see Table 1) are contained within the matrix space. All communication with the cell cytosol occurs through metabolites which move across the inner mitochondrial membrane. The permeability of the inner membrane is highly selective even towards uncharged molecules of low molecular weight (greater than approximately 100 daltons) and all ionic species. This selectivity allows the mitochondrion to build and maintain a matrix environment different from that of the cytosol with respect to metabolite and ion concentrations. The restricted permeability of the inner membrane also means that the flow of metabolites between the mitochondrial matrix and the cytosol requires specific transport mechanisms (carrier proteins) which can be an important site of cellular metabolic regulation (Chappel and Crofts, 1966; Chappel and Haarhoff, 1967; Klingenberg, 1970; Meijer and Van Dam, 1974; LaNoue and Schoolwerth, 1979). The matrix space behaves *in vitro* as a nearly ideal osmometer obeying Henry's law. The matrix shrinks as the concentrations of osmotically active species in the suspending medium are increased and swells as the concentrations are decreased (Tedeschi and Harris, 1955; Lehninger, 1962). The isolation of mitochondria with maximum retention of their morphology (as seen by electron microscopy) requires media of 0.6 to 0.8 osmolar while maximum biochemical activity requires media of 0.25 to 0.35 osmolar. The inner mitochondrial membrane also provides a structural template for the organization of the mitochondrial respiratory chain and its associated phosphorylation reactions. The respiratory chain is an integral part of the membrane and is organized in a manner that facilitates the rates and specificities of the reactions involved.

The Outer Mitochondrial Membrane and the Intermembrane Space

The outer mitochondrial membrane forms a relatively smooth shell around the inner membrane. Its surface area is much less than that of the inner membrane due to the many invaginations of the latter. The outer membrane is believed to be fully permeable to molecules of less than approximately 1000 daltons but not to larger molecules. Thus the intermembrane space serves as a compartment in which soluble enzymes (such as adenylate kinase) can be separated from the cytosol and yet have free access to their substrates (such as ATP, ADP and AMP). When isolated mitochondria are suspended in a medium which is hypotonic with respect to the contents of the matrix space, the inner mitochondrial membrane can swell until the outer membrane is broken (due to the smaller surface area of the outer membrane). The inner membrane remains undamaged and does not become leaky to the contents of the matrix.

Transport across the mitochondrial membrane

The usual environment of the mitochondrion *in vivo* is an ionic surrounding with potassium (80–150 mM) and sodium (20–70 mM) as chief cations and chloride (80–150 mM) and bicarbonate (5–20 mM) as the main anions. Of considerable physiological importance are three other ions present in the cytosol in much smaller concentrations (the values given represent the concentrations of the free species and not total contents); magnesium (1×10^{-3} M), calcium ($\sim 10^{-6}$–10^{-7} M) and phosphate (2–6×10^{-3} M). The cytosol also contains relatively high concentrations of adenine nucleotides and various metabolites (for example lactate and pyruvate). Determination

of the ionic composition of the mitochondrial matrix space in intact cells *in vivo* is an experimentally difficult problem. Electron probe analyses of frozen cell sections show that there are no major gradients of K^+ and Na^+ between the cytosol and the mitochondrial matrix but that intramitochondrial $[Ca^{2+}]$ is higher than that in the cytosol (Somlyo *et al.*, 1979).

The inner mitochondrial membrane, which provides a barrier to the movement of small molecules, complies with the physical laws that apply to the flow of substances across any semipermeable membrane. Since a number of chapters in this book deal with transport of ions and their inhibitors, we considered it worthwhile to remind the reader about certain basic principles of transport which could guide him through the maze of specific information, very often complicated and confusing.

(a) *Some basic principles involved in transport across semipermeable membranes*

Introduction of two different solutions on the opposite side of a semipermeable membrane initiates movement of permeable solutes between the two compartments. These movements are subject to three important physical considerations: osmotic pressure, electrical gradients and activity gradients.

Osmotic pressure: The flow of solute across a membrane which is permeable to solvent (water) must, in the steady state, occur with no net increase in the osmolarity (concentration of impermeant species) of the media on either side of the membrane. Any change in osmolarity of the medium on one side of the membrane relative to the other gives rise to a change in either the osmotic pressure on the membrane or the compartment volume. Thus steady state is achieved only when the net flow of any osmotically active solute across the membrane in one direction is exactly balanced by the net flow of other osmotically active solutes across the membrane in the opposite direction.

Electrical gradients: It is a necessary condition that the sum total of positive charges on one side of a membrane equals the sum total of negative charges on the same side. Any unbalance of these charges gives rise to an electrical potential across the membrane and even very small amounts of charge give rise to large electrical potentials across distances corresponding to the thickness of natural membranes. Since in the steady state the total number of positive and negative charges must balance, it follows that if an ion with a net charge is to cross the membrane, it must either do so in exchange for another ion of the same charge or be accompanied by an ion having an equal but opposite charge. For the case of simple diffusion this is expressed by the Goldmann–Hodgkin–Katz equation:

$$v = \frac{RT}{F} \ln \left[\frac{\Sigma(P_j C_{je})^+ + \Sigma(P_j C_{ji})^-}{\Sigma(P_j C_{ji})^+ + \Sigma(P_j C_{je})^-} \right] \tag{1}$$

where P_j is the permeability of the ionic species. The sign of the membrane potential is determined by the individual ion concentration gradient and the ion permeabilities; its magnitude is thus determined by the individual conductances. At equilibrium ($\Delta G = 0$) distribution of a permeable ion is determined by the transmembrane electrical potential:

$$\Delta G = nFE + RT \ln \frac{C_i}{C_e} \tag{2}$$

where E is the transmembrane electrical potential. When the system is not at equilibrium the ion will diffuse across the membrane in the direction of a negative free energy change unless the membrane is completely impermeable to that particular ion. This is true, however, only if the membrane does not have an active transport system for the ion and a source of energy for active transport process.

Activity (concentration) gradients

The presence of an activity gradient across a biological membrane provides a driving force for the transport of compounds.

$$\Delta G = RT \ln \frac{A_i}{A_e} \tag{3}$$

where the free energy change (ΔG) associated with the movement of one mole of compound from outside to inside is related to the activities of that compound inside (A_i) and outside (A_e). For most biological compounds the activity coefficient is similar on both sides of the membrane and the approximate expression

$$\Delta G = RT \ln \frac{C_i}{C_e} \tag{4}$$

holds where C_i and C_e are the internal and external concentrations. Unless other forces (osmotic, electrical, active transport, etc.) are present, the compound will move down its concentration gradient.

Coupled (exchanged) transport systems: When exchange mechanisms are involved, the reaction is

$$aA_e^n + bB_i^m \rightleftharpoons aA_i^n + bB_e^m \tag{5}$$

where a moles of compound A with electrical charge n is exchanged for b moles of compound B with charge m. Assuming an electroneutral exchange (net flow of electrical charge is zero) the free energy change for the transport of compounds A and B is the sum of that due to the concentration gradients.

$$\Delta G = RT \ln\left(\frac{[A_i]}{[A_e]}\right)^a \left(\frac{[B_e]}{[B_i]}\right)^b. \tag{6}$$

When $a \neq b$ this coupled transport system would also give rise to osmotic changes unless a separate system exists to restore osmotic balance. In most eukaryotic systems (except in plants) osmotic pressure does not contribute a significant force.

The transport process described in equation 6 may occur such that $a \times n \neq b \times m$ and the exchange mechanism results in net transport of electrical charge across the membrane. In this case the transport process is referred to as electrogenic and the transmembrane electrical potential can be an important force. The overall driving force for the transport process is the sum of the ΔG values for the activity and electrical gradients

$$\Delta G = RT \ln\left(\frac{[A_i]}{[A_e]}\right)^a \left(\frac{[B_e]}{[B_i]}\right)^b + (an - bm)FE \tag{7}$$

where E is the transmembrane potential and $(an - bm)$ is the net electrical charge moved across the membrane when a moles of compound A is exchanged for b moles of compound B. By convention when the inside of a vesicle is negatively charged with respect to the outside of the vesicle, the membrane potential is negative.

(b) *Transport mechanisms across membranes*

Transport of molecules (or substances) across the membrane occurs through three basic mechanisms:

1. Simple diffusion through pores or holes in the membrane.
2. Facilitated diffusion involving binding sites in the membrane.
3. Active transport.

Diffusion is a process in which movement of molecules is determined exclusively by thermal molecular motion. It follows from this definition that diffusion across a membrane consists of two unidirectional velocities (fluxes) which are independent of each other but directly dependent on the starting concentration of the diffusing substance. The rate of net movement i.e. the difference between the two unidirectional fluxes is proportional to the difference of activities (or concentrations) on the two sites of the membrane (or in case of ions, to the difference in electrochemical activities of diffusing ions). This relationship is expressed by Fick's law:

$$\frac{d_n}{d_t} = -DA\frac{dc}{dx} \tag{8}$$

where d_n/d_t denotes the number of molecules transferred per unit time, D is diffusion coefficient, A is the area, c is the concentration and x the distance. Only a few molecules enter the mitochondrion by this mechanism. Oxygen and water are probably the main ones.

Carrier-mediated (or facilitated) *diffusion* also operates on an existing electrochemical gradient of the permeant species and does not lead to a net movement against the thermodynamic gradient. The net movement is always *down* the gradient and all the energy for the transport is derived from the chemical gradient of the transported substance itself. It contains an additional process in which the penetrating molecule (or ion) forms a complex with a membrane component (carrier) which then 'transfers' the bound species across the membrane. The rate of penetration is proportional to concentration of the permeating substance at low concentrations but reaches a saturation value and decreases as saturation of the binding site(s) is exceeded. This is expressed in Michaelis-Menten kinetics which the carrier-mediated processes obey:

$$v = V_{\max}\left[\frac{K_M(S_e - S_i)}{(S_e + K_M)(S_i + K_M)}\right]. \tag{9}$$

The rate of penetration may be markedly decreased by the presence of molecules structurally analogous to the permeant species (competition for the binding site), and also by substances differing chemically from the permeant species (non-competitive inhibitors). Carrier-mediated facilated diffusion can be classified either as exchange diffusion or as counter-transport. Exchange diffusion describes the situation in which the carrier is unable to move a species without the membrane simultaneously transferring another species in the opposite direction. Thus no net transport of substrate is possible without simultaneous countermovement of a second substrate. In this process, movement of one substance can show net transport against its electrochemical gradient if the net flow of the second substance is down its electrochemical gradient. The positive free energy change (ΔG) associated with movement of one substance against its electrochemical gradients is overcome by the negative ΔG associated with the flow of the other substance down its electrochemical gradient; the necessary coupling of the two flows being provided by their sharing of the same facilitated diffusion system. There are a number of substances that enter and/or leave mitochondrion through facilitated diffusion or exchange diffusion. Specific proteins which catalyze transport of anions have been identified in the mitochondrial inner membrane and are dealt with in this volume by Bryła (anion substrate translocators) and Fonyó (phosphate transporter).

Active transport involves an energy dependent transport of a molecule or ion against its electrochemical gradient and thus requires input of energy. It follows from this definition that the ratio of the undirectional fluxes is *not* identical with the ratio of activities and that the K_m values for the transported ions on both sides of the membrane are *not* identical. Active transport systems can be divided for the sake of simplicity into four groups, the basis of classification being the source of energy necessary to maintain the system. These groups are:

1. Light-energy dependent conformation driven reactions as exemplified by Halobacterium halobium proton pump (Osterhelt and Stoeckenius, 1971; Blaurock and Stoeckenius, 1971).
2. Redox-reaction dependent systems in which the energy necessary for uphill transfer of substances is provided by electron transport.
3. ATP-dependent transport systems in which the energy is provided by ATP hydrolysis.
4. Co-transport systems utilize the hydrolysis of ATP to ADP and inorganic phosphate to provide the energy required. In a case where ions are being transported against their concentration gradient, the pumps may be electrogenic in character and generate a membrane potential. The maximal voltage that a given electrogenic pump can sustain depends on the stoichiometry of pump action and the general conductance

(i.e. the degree of 'leakiness' of the membrane for the ions present). The principal ions which are known to be transported against their concentration gradient are sodium, potassium, and calcium in animal cell membrane of nerve, muscle, and epithelial tissue (Kernan, 1970; Ritchie, 1971; Thomas, 1972); chloride in some epithelial membranes (Kernan, 1970; Rehm, 1966) and in plants cells (Saddler, 1970); Ca^{2+} in sarcoplasmic reticulum (Ebashi, 1960, 1961; Hasselbach and Makinose, 1961; Weber *et al.*, 1966) and hydrogen ions in the gastric mucosa (Rehm, 1972; Sachs *et al.*, 1978) and in plant and fungal cells (Kitasato, 1968; Slayman *et al.*, 1973). It has been postulated (Mitchell, 1966; Skulachev, 1975) that in mitochondria there is a reversible ATP-coupled H^+ pump and an electrochemical gradient of H^+ ions which are produced by the mitochondrial respiratory chain and utilized for the synthesis of ATP.

Principles of energetics of electron transport and oxidative phosphorylation

Any oxidation-reduction reaction may be represented

$$aA_r + bB_o \rightleftharpoons aA_o + bB_r \tag{10}$$

where the subscripts o and r indicate the oxidized and reduced forms respectively and a and b are the stoichiometrics of components A and B. This reaction is a complete electrical cell and is conventionally analysed as the sum of two half cell reactions

$$A_o + ne^- \rightleftharpoons A_r \tag{11}$$

$$B_o + ne^- \rightleftharpoons B_r \tag{12}$$

by relating each to a standard hydrogen electrode. The voltages which would be developed by reactions of A and B relative to a standard hydrogen electrode (E_h) are expressed:

$$E_{hA} = E_{mA} + \frac{RT}{nF} \ln \frac{A_o}{A_r} \tag{13}$$

$$E_{hB} = E_{mB} + \frac{RT}{nF} \ln \frac{B_o}{B_r} \tag{14}$$

where E_{mA} and E_{mB} are the characteristic half-reduction potentials for the two redox components, n is the number of reducing equivalents accepted per mole of reactant, and R, T and F are the gas constant, absolute temperature and Faraday constant respectively. The electrical gradient (ΔE) which can be generated by transfer of reducing equivalents from A to B is the difference in their calculated E_h values ($E_{hB} - E_{hA}$):

$$\Delta E = E_{hB} - E_{hA} = E_{mB} - E_{mA} + \frac{RT}{nF} \ln \frac{B_r A_o}{B_o A_r}. \tag{15}$$

The process of oxidative phosphorylation utilizes the ability of the oxidation-reduction reactions to provide the energy (work) required to synthesize ATP from ADP and *Pi*. The Gibbs free energy change (ΔG) associated with the transfer of reducing equivalents

$$\Delta G = -nF\Delta E \tag{16}$$

is a measure of the maximum work which can be provided by the redox reactions and which can be utilized to drive endergonic reactions such as ATP synthesis or ion transport. In order to evaluate ΔG it is necessary to know both the characteristic half reduction potentials (E_m values) for the respiratory chain components, and to measure the ratio of the oxidized and reduced species of the components for the metabolic conditions of interest.

In the overall process of oxidative phosphorylation the negative free energy change for transfer of reducing equivalents is utilized to synthesize ATP from ADP and inorganic phosphate (*Pi*)

$$\text{ADP} + Pi \rightarrow \text{ATP} \tag{17}$$

for which the free energy requirements are expressed

$$\Delta G_{ATP} = \Delta G^{\circ\prime}_{ATP} + RT \ln \frac{[ATP]}{[ADP]\,[Pi]} \tag{18}$$

where $\Delta G^{\circ\prime}$ is the standard free energy of synthesis of ATP at pH 7.0 and has a value near 7.6 kcal/mole at a free Mg^{2+} concentration of 1 mM. In order for net synthesis of ATP to occur the free energy change for the coupled reaction

$$aA_r + bB_o + c\text{ADP} + cPi \rightleftarrows aA_o + bB_r + c\text{ATP} \tag{19}$$

must be negative. From equations 16 and 18 the inequality is expressed:

$$-nF\Delta E + \Delta G^{\circ\prime} + RT \ln \frac{[ATP]}{[ADP]\,[Pi]} \leqslant 0. \tag{20}$$

A similar relationship must hold for any proposed intermediate process in oxidative phosphorylation. For such an intermediate, the free energy change for its formation during oxidative phosphorylation, ΔG_i, must fulfill two inequalities

$$-nF\Delta E + \Delta G_i \leqslant 0 \quad \text{and} \quad \Delta G_i + \Delta G_{ATP} \leqslant 0 \tag{21}$$

and these combine to give:

$$-nF\Delta E \leqslant -\Delta G_i \leqslant -\Delta G_{ATP}. \tag{22}$$

The mechanism of oxidative phosphorylation is yet unknown, but extensive efforts are being made to determine if proposed intermediates, such as the proton electrochemical gradient (Mitchell, 1966; Skulachev, 1975), fulfill this requirement.

The mitochondrial respiratory chain: A definition

The most unique and characteristic set of enzymes that the mitochondrion possesses are the respiratory chain components. The mitochondrial respiratory chain, in the most simple terms, is a multienzyme complex which accepts reducing equivalents from donor substrates and transfers them in a sequence of oxidation-reduction reactions to molecular oxygen. This transfer of the reducing equivalents takes place down an electrochemical potential gradient and the energy set free in the exergonic redox reactions can be converted into a form suitable for use in ATP synthesis. *In vivo*, the coupling between the transfer of electrons and phosphorylation of ADP is obligatory and the overall process is called oxidative phosphorylation.

The constituents of the respiratory chain include chemically diverse oxidation-reduction components: hemeproteins, copperproteins, iron-sulfur proteins and flavoproteins all of which form a part or are intimately associated with the inner mitochondrial membrane. The respiratory chain is not considered to include the mitochondrial dehydrogenases which are soluble in the matrix space (such as those for malate, isocitrate, glutamate, α-ketoglutarate and pyruvate, etc.). On the other hand, the NADH dehydrogenase (a flavin-containing enzyme which oxidizes NADH produced by the matrix dehydrogenases) and succinate dehydrogenase (a flavin-containing enzyme which catalyzes oxidation of succinate to fumarate) are both tightly bound to the mitochondrial inner membrane and considered as integral parts of the respiratory chain. Moreover, owing to the obligatory coupling between the redox reactions and phosphorylation reactions the pathway which transfers the reducing equivalents must remain in close structural and functional contact with the enzymes which lead to the synthesis of ATP. Thus, the ATP-ase complex is intimately associated with the mitochondrial respiratory chain.

Historical Sketch of the Development of the Respiratory Chain

The idea that myohematins pigments, originally discovered by McMunn (1884, 1886, and 1887), are intimately involved in cellular respiration was put forward by David Keilin in the early 20th century. Using a microspectroscope Keilin (1925) was able to

observe disappearance and reappearance of specific spectral absorption bands upon reversible oxidation and reduction. These same components could be identified by their spectral properties in diverse biological groups such as mammals, amphibians, insects, yeasts, even bacteria and plants, which indicated their universality. Keilin termed the pigments—the cytochromes—and recognized them as hemoproteins, proteins in which the prosthetic group was an iron porphyrin complex (heme). On the basis of the different absorption maxima in the visible region of the spectrum the cytochromes were designated as *a*, *b* and *c*. In 1925 Keilin noted that addition of urethane to a particular preparation from heart muscle resulted in reduction of cytochrome *b* and oxidation of cytochrome *c* and *a*. This finding, together with the evidence that isolated cytochrome *c* was not the component which reacts with molecular oxygen left little doubt that the sequence

$$\text{substrate} \rightarrow \text{cytochrome } b \rightarrow \text{cytochrome } c \rightarrow \text{cytochrome } a \rightarrow O_2$$

was responsible for the transport of reducing equivalents (Keilin 1925, 1929, 1930, 1966; Keilin and Hartree 1939).

The presence of such a sequence of oxidation-reduction reactions as an integral part of the enzymatic system responsible for cellular respiration effectively united Wieland's concept of dehydrogenation (Wieland, 1912) and Warburg's ideas of biological oxidations (Warburg, 1924, 1926). In the late 1920's Warburg postulated (Warburg, 1924, 1926) that the transfer of hydrogen atoms to molecular oxygen was mediated by an oxidase with a hemoprotein nature ('Atmungsferment'). The existence of such oxidase was implicated by the characteristic, competitive with respect to oxygen, inhibition of cellular respiration by carbon monoxide and its reversal by light (Warburg and Negelein, 1929). Warburg initially rejected the important discovery of the respiratory chain by Keilin (1925, 1929) as Keilin was unable to observe photodissociation of CO-compound of reduced cytochrome a_3. This gave rise to a controversy concerning the relationship between Keilin's cytochromes and Warburg's 'Atmungsferment'. It was not until the late 1930's that Keilin and Hartree (1939) described the absorption bands and the properties of cytochrome a_3 and its carbon-monoxide compound and accepted its identity with Warburg's 'Atmungsferment'. It remained to Chance (Chance, 1952; Castor and Chance, 1955) however, to demonstrate the photodissociation of CO-compound of reduced cytochrome a_3 in a manner predicted by Warburg's experiments.

Thus in the early 1940's the respiratory chain was composed of cytochrome *c* (soluble and partially purified), cytochrome *b* and cytochrome oxidase which consisted of two components, cytochrome *a* and a_3, one of which (cytochrome a_3) was capable of binding ligands. Keilin (1929, 1930) identified three types of heme: heme *c*, protoheme IX and heme *a* as the prosthetic groups for cytochrome *c*, cytochrome *b* and cytochrome $a + a_3$, respectively.

In 1940 Yakushiji and Okunuki working with detergent extracts of a particulate preparation from heart muscle noted the presence of a new pigment of a hemoprotein nature. They called this cytochrome c_1. Their observation was confirmed by Slater (1949) but the latter attributed the measured absorption spectrum to denatured protein protohemochromogen which contaminated his preparation rather than to a new respiratory pigment. In the same year Keilin and Hartree (1949) observed in heart muscle preparation and yeast cells the presence of a hemoprotein (termed by them cytochrome *e*), with spectral properties identical to that of cytochrome c_1. Although Chance and Pappenheimer (1954) argued that cytochrome *e* represented cytochrome b_5 contamination, later studies of Keilin and Hartree (1955) and of Estabrook (1955) established conclusively the existence of cytochrome *e* and its identity with cytochrome c_1. Acknowledging the priority of the Japanese investigators, Keilin and Hartree (1955) abandoned the term cytochrome *e* and adopted the designation cytochrome c_1. The direct participation of cytochrome c_1 in electron transport and its position in the respiratory chain between cytochrome *b* and *c* was established by demonstrating that in a cytochrome *c* extracted preparation cytochrome c_1 was readily reduced by substrate but its subsequent reoxidation by molecular oxygen was severely inhibited.

Our understanding of the role of cytochrome *b* in the respiratory chain has had a long