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# Chemiosmotic Theory

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A0005

P0005 Mitochondria transform the chemical energy derived from food and body stores into ATP by a process called oxidative phosphorylation. The chemiosmotic theory begins by describing the mechanism of coupling between substrate oxidation and phosphorylation. It goes on to describe the membrane properties that are required in order for mitochondria to provide ATP to the cell and, indeed, to survive within the cell. The chemiosmotic theory was presented as a hypothesis far in advance of experimental evidence, and it stands as a monument to the scientific method. For this extraordinary achievement, Peter Mitchell was awarded the Nobel prize in chemistry in 1978.

## S0005 Basic Chemiosmotic Theory

### S0010 MITOCHONDRIAL STRUCTURE

P0010 Mitochondria are small, vesicular organelles. The internal aqueous compartment is called the *matrix*, which contains the enzymes of the Krebs tricarboxylic acid cycle. The matrix is enclosed by a highly folded, insulating membrane called the inner membrane, which contains the enzymic machinery of oxidative phosphorylation. The inner membrane is separated from the cytosol by a more permeable outer membrane, and the aqueous compartment between the inner and outer membranes is called the intermembrane space.

### S0015 THE FOUR POSTULATES

P0015 Peter Mitchell proposed that nature uses protonic batteries to drive ATP synthesis and that biological energy conservation is essentially a problem in membrane transport, as diagrammed in [Figure 1](#). The chemiosmotic theory consists of four postulates.

P0020 1. The inner membrane contains electron transport enzymes which are vectorially oriented so that the energy of electron transport drives ejection of protons outward across the membrane. The energy of substrate oxidation is thereby converted to and stored as a proton electrochemical potential gradient, called the protonmotive force.

P0025 2. The inner membrane contains a reversible, proton-translocating ATPase, which is also vectorially oriented so that the energy of ATP hydrolysis will drive protons outward across the inner membrane. The ATPase is reversible, so that protons driven inward through the enzyme by the redox-generated protonmotive force will cause ATP synthesis.

P0030 3. The inner membrane must have a low diffusive permeability to ions in general and to protons in particular. Otherwise, ion leaks would short-circuit the protonmotive batteries, and ATP would not be synthesized.

P0035 4. The inner membrane was postulated to contain exchange carriers in which anion entry is effectively coupled to proton entry. This provides a thermodynamically favorable pathway for substrate anions to reach enzymes within the electronegative matrix. The membrane was also postulated to contain exchange carriers in which cation exit is coupled to proton entry. This provides a thermodynamically favorable pathway for removal of cations that entered the matrix by diffusion down the very large electrical gradient caused by outward proton pumping.

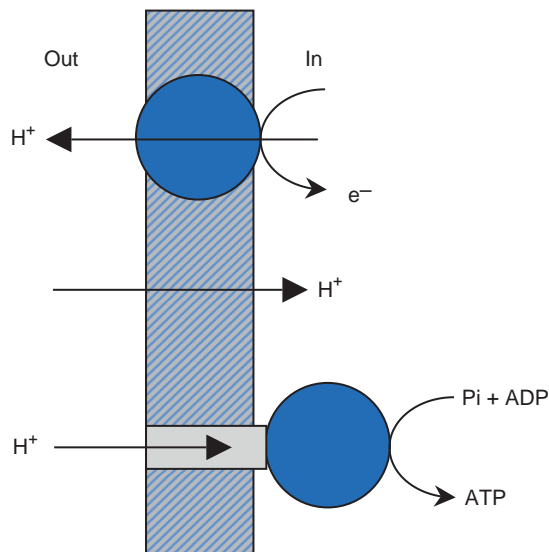
## S0020 First Postulate – Respiration and the Electron Transport System

### S0025 ELECTRON TRANSPORT

P0040 NADH and succinate arising from the tricarboxylic acid cycle are oxidized by the electron transport chain as diagrammed in [Figure 2](#). The structure–function of the electron transport enzymes is discussed in other chapters.

### S0030 PROTONMOTIVE FORCE

P0045 Electron transport through complexes I, III, and IV is coupled to electrogenic proton ejection across the inner membrane. The protonmotive force ( $\Delta p$ ) is the free energy per mol for moving protons outward across the membrane. It is simply the sum of the work done against the electrical force and the work done against the proton



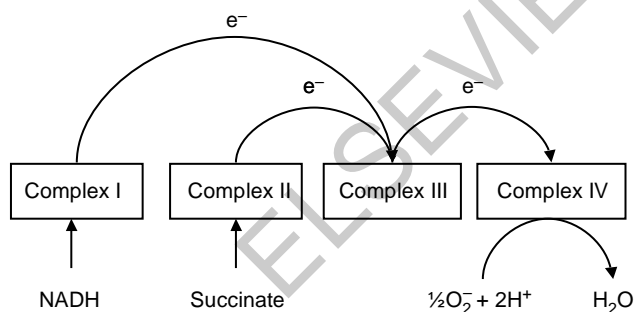
F0005 **FIGURE 1** Coupling of electron transport with ATP synthesis. The chemiosmotic theory states that vectorial enzymes of electron transport and ATP synthesis are coupled “indirectly” via the protonmotive force across the inner membrane of mitochondria. In order for oxidative phosphorylation to be coupled by this mechanism, the membrane must have a low permeability to protons (postulate 4).

concentration difference.  $\Delta p$  is defined as the electrochemical proton gradient divided by the Faraday constant ( $\Delta\mu_{H^+}/F$ ); therefore

$$\Delta p = \Delta\psi - Z\Delta pH \quad (1)$$

Q1

where  $Z \equiv (RT \ln 10)/F = 59 \text{ mV}$  at  $25^\circ\text{C}$ , and  $\Delta\psi$  is the membrane potential (inside minus outside).  $\Delta\psi$  and  $\Delta pH$  can be estimated from equilibrium distributions of cationic dyes and weak acids, respectively. It is customary in bioenergetics to drop the negative signs of  $\Delta\psi$  and  $\Delta p$ . Commonly observed values in isolated,



F0010 **FIGURE 2** Electron transport chain in mitochondria. Complex I (NADH-coenzyme Q reductase) and Complex II (Succinate coenzyme Q reductase) feed electrons into coenzyme Q at Complex III (Coenzyme Q cytochrome c reductase), then onto cytochrome c, which delivers them to Complex IV (Cytochrome c oxidase). At Complex IV, molecular oxygen is reduced by the electrons to water. Electrogenic proton ejection occurs via complexes I, III, and IV.

nonphosphorylating mitochondria are  $190 \text{ mV}$  for  $\Delta\psi$ ,  $0.3$  units for  $\Delta pH$ , resulting in a  $\Delta p$  of  $\sim 208 \text{ mV}$ .

## STOICHIOMETRIES

S0035

In the final step of electron transport, the dioxygen molecule ( $\text{O}_2$ ) is reduced to water by four electrons ( $e^-/O = 2$ ). When a pair of electrons moves from NADH to oxygen, it is estimated that ten protons are ejected across the inner membrane ( $H^+/O = 10$ ).

## RESPIRATORY CONTROL

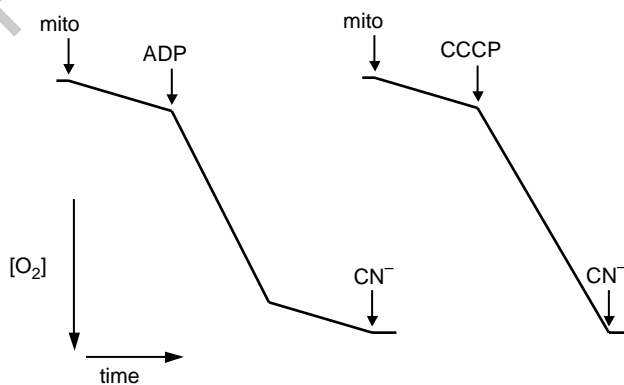
S0040

Respiration can readily be measured as oxygen uptake by isolated mitochondria. The typical traces in Figure 3 illustrate the principle of respiratory control, which is that respiration increases if the proton back-flux across the inner membrane is facilitated, either through the ATP synthase or by proton-translocating drugs or proteins.

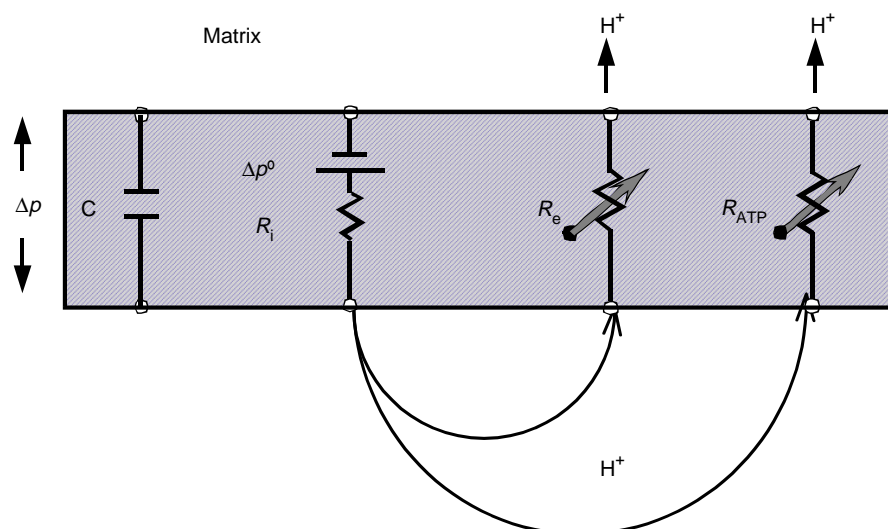
## THE PROTONMOTIVE CIRCUIT

S0045

The chemiosmotic theory identifies the electron transport system (ETS) as a protonmotive cell, the behavior of which is identical to the well-known behavior of electromotive circuits, such as is shown in Figure 4. We note four salient aspects of this circuit: (1) The electron current is measured as respiration, as shown in Figure 3. (2) The current is determined entirely by the external resistances, and the battery will deliver increased current only when  $R_c$  or  $R_{ATP}$  are decreased.



F0015 **FIGURE 3** Respiratory control in mitochondria. Mitochondria are added to a closed vessel containing an oxygen electrode and a medium containing phosphate and substrates to support respiration. A slow rate of oxygen consumption is observed, which is due primarily to proton leak across the inner membrane. When ADP is added, respiration accelerates until most of the ADP is consumed, after which it returns to the control rate. The protonophore CCCP accelerates respiration by catalyzing proton back-flux across the inner membrane. Respiration is stopped by addition of cyanide, which inhibits cytochrome c oxidase.



F0020 **FIGURE 4** Circuit diagram of the mitochondrial electron transport system (ETS). In the diagram, the battery symbol corresponds to the ETS, connected across the membrane through an internal resistance,  $R_i$ .  $C$  is the capacitance of the membrane.  $\Delta p^\circ$  corresponds to the free energy drop when two electrons pass from the input redox couple to oxygen.  $R_e$  is the sum of cation and proton leak resistances and those of the futile cation cycles necessary to regulate mitochondrial physiology. Proton back-flux through the ATP synthase is designated by the element containing  $R_{ATP}$ . If conductance through both of these back-flux pathways were zero, there would be no respiration, no proton pumping, and  $\Delta p$  would equal  $\Delta p^\circ$ , the open-circuit voltage.

(3) The battery will respond the same whether current is drawn through  $R_e$  or  $R_{ATP}$ . (4) As increasing current is drawn from the battery, the voltage will decrease, due to the internal resistance,  $R_i$ . Thus, respiration is “driven” by the free energy contained in the redox drop, and it is “controlled” by the proton back-flux through leak pathways and the ATP synthase.

### S0050 BEHAVIOR OF THE PROTONMOTIVE CIRCUIT

P0065 The experiment in Figure 5 shows how  $\Delta p$  varies when electron current is progressively increased by adding a protonophore that decreases external resistance ( $R_e$ ) to  $H^+$  ions. The resulting increase in respiration causes  $\Delta p$  to fall gradually until the  $V_{max}$  of the ETS is reached. What is being measured in such experiments is evident from the circuit diagram of Figure 4:

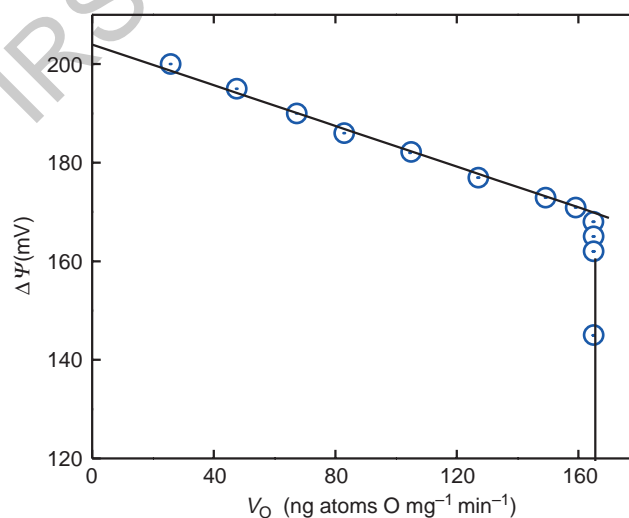
$$\Delta p = \Delta p^\circ - R_i \times V_O \quad (2)$$

where  $V_O$  is the respiration rate. The slope,  $R_i$ , is the internal resistance of the ETS, representing the weighted sum of frictional coefficients of the reactions leading to proton ejection. The intercept is  $\Delta p^\circ$ , the theoretical open-circuit voltage of the system, whose value is given by

$$\Delta p^\circ = (2/n_H)\Delta E \quad (3)$$

where  $n_H$  is the  $H^+/O$  stoichiometry, and  $\Delta E$  is the redox span being studied. The total redox potential,  $\Delta E$ , for a

pair of electrons moving from NADH/NAD<sup>+</sup> to oxygen is  $\sim 1.16$  V. Therefore, if  $n_H$  is 10,  $\Delta p^\circ = 232$  mV. Decreasing  $R_e$  to increase proton back-flux may be achieved by ionophores, by uncoupling protein, or by



F0025 **FIGURE 5** Dependence of protonmotive force on electron transport rate. Membrane potential ( $\Delta\psi$ ) of rat liver mitochondria is plotted versus respiration rate ( $V_O$ ), which was varied by adding the protonophoretic uncoupler, CCCP. Respiration was measured using a standard Clark electrode.  $\Delta\psi$  was determined from the distribution of tetraphenylphosphonium cation. The slope of the curve,  $R_i$ , is 0.35, the intercept is 210 mV, and  $V_{max}$  is  $\sim 160$  ng atom O  $\text{min}^{-1} \text{mg}^{-1}$ . These are typical values for rat liver mitochondria respiring on succinate. The pH gradient was 0.3 and assumed to be invariant with increased respiration. Therefore,  $\Delta p^\circ$  is  $\sim 228$  mV.

futile  $\text{Ca}^{2+}$  or  $\text{K}^+$  cycling. Decreasing  $R_{\text{ATP}}$  to increase proton back-flux may be achieved by adding ADP and phosphate so that current is drawn via the ATP synthase. Careful measurements show that all methods of increasing respiration yield points that fall on the same battery curve as illustrated in Figure 5.

## S0055 Second Postulate – The ATP Synthase

P0070  $\Delta p$  formed by the ETS is used to drive ATP synthesis via a remarkable series of steps. First, a proton binds to one of the 10–14 C subunits of the  $F_0$  complex. This induces a conformational change that causes the C ring to rotate, after which the bound proton is released into a channel that carries it into the matrix. In this fashion,  $\Delta p$  is transduced into a rotary mechanical force. The rotation of the C ring, in turn, drives the rotation of the attached  $\gamma$ -subunit, and rotation of  $\gamma$  induces conformational changes in the catalytic sites of the three  $\beta$ -subunits of the  $F_1$  head group of the ATP synthase. These catalytic sites exist in three different conformations, corresponding to the three faces presented by the end of the  $\gamma$  subunit. If one proton is associated with each step of the cycle as it occurs on  $F_1$ , then the  $\text{H}^+$ /ATP stoichiometry would be 3 for ATP synthesis. On the other hand, if a complete revolution of the C ring is required for ATP synthesis, and if there are 12 C subunits in  $F_0$ , then the stoichiometry would be 4.

## S0060 Third Postulate – Ion Leaks and the Permeability Barrier

P0075 Notwithstanding the low diffusive permeability of the inner membrane, cation, and proton leaks occur at significant rates, and they are physiologically important. Inward  $\text{K}^+$  leak causes matrix swelling, and inward proton leak contributes to the basal metabolic rate. Moreover, nature has engineered the uncoupling proteins to increase proton leak under certain physiological circumstances.

## S0065 ION LEAKS IN MITOCHONDRIA

P0080 Diffusive transport of ions obeys the same laws that govern transport of nonelectrolytes across thin membranes. The rate is proportional to the concentration difference, and the proportionality constant (the permeability coefficient) is a function of the energy barrier that must be crossed during transport. The ionic charge adds a new complexity that derives from the long-range effects of the electric field on the local free energy of the diffusing ions. An ion diffusing across the inner

membrane of mitochondria must cross an energy barrier whose maximum is located at the center of the membrane, and only those ions having sufficient energy to reach this peak will cross to the energy well on the opposite side. Net flux will therefore be proportional to the differential probability of getting to this peak from either side. This probability is given by the Boltzmann function,  $\exp(-\Delta\mu_p/RT)$ , where  $\Delta\mu_p \equiv \mu_p - \mu_{aq}$  is the Gibbs energy of the ion at the peak ( $p$ ) relative to its value in the aqueous energy well at the surface of the membrane ( $aq$ ). These considerations lead to the following expression for diffusive flux of cations across thin biomembranes:

$$J = fxP(C_1e^{u/2} - C_2e^{-u/2}) \quad (4)$$

where  $u$  is the reduced voltage ( $zF\Delta\psi/RT$ ),  $C_1$  and  $C_2$  are bulk aqueous concentrations,  $f$  is the surface partition coefficient (energy well/bulk), and  $P$  is the permeability constant, given by

$$P \equiv ke^{-\Delta\mu_p^0/RT} \quad (5)$$

The factor 1/2 in the exponents of eqn. (4) arises from the fact that the maximum energy barrier is found at the midpoint of the membrane.

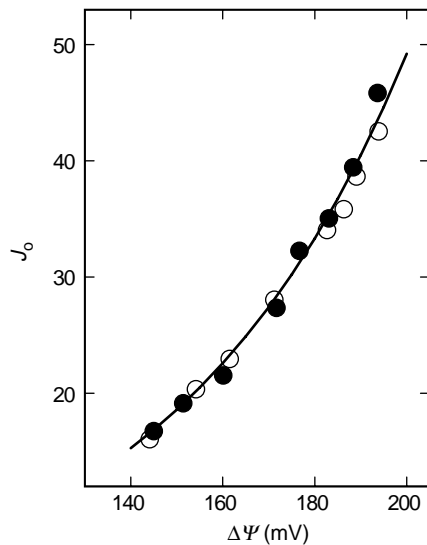
The second term in eqn. (4) represents back-flux of cations from the matrix and becomes negligible at the high values of  $\Delta\psi$  maintained by mitochondria under physiological conditions. Thus, eqn. (4) reduces to a simple exponential function of  $\Delta\psi$ :

$$J = fPC_1e^{u/2} \quad (6)$$

Eqn. (6) emphasizes the point that ion flux at high potentials is not affected by the concentration gradient across the membrane. Figure 6A contains data showing that proton leak is in good agreement with eqn. (6), and the flux–voltage plots in Figure 6B show that diffusion of  $\text{TEA}^+$  (tetraethylammonium ion) and  $\text{H}^+$  (hydronium ion) across the inner membrane behave identically with respect to their voltage-dependence.

## UNCOUPLING PROTEINS

Uncoupling proteins (UCPs) are the exception that proves the rule of the inner membrane permeability barrier. Nature devised the UCPs to intentionally short-circuit the inner membrane in order to dissipate energy and generate heat instead of ATP. UCP1 is expressed solely in brown adipose tissue, where it plays a major role in providing heat to hibernating animals and mammalian newborns. The human genome contains at least three additional UCPs, designated UCP2–4. UCP2 is ubiquitously expressed in mammalian tissues; UCP3 is expressed primarily in glycolytic skeletal muscle in humans; and UCP4 is expressed in brain. The roles of UCP in non-thermogenic tissue are uncertain but may



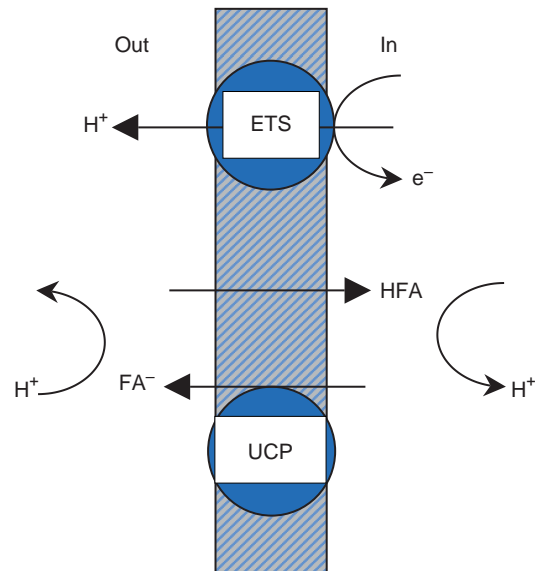
**FIGURE 6** Proton and cation diffusion across the inner membrane. (A) Proton flux. The semilogarithmic plot shows that  $H^+$  leak is exponential with voltage, and the slope of  $\ln J_H$  vs.  $F\Delta\psi/RT$  is  $1/2$ , in agreement with eqn. (6). The intercept of such plots gives  $J_o \equiv fP[C]_o$ , where  $[C]_o$  is the aqueous concentration, and  $f$  and  $P$  are defined in the text. (B) Tetraethylammonium ( $TEA^+$ ) and  $H^+$  flux. Fluxes were normalized to their respective values of  $J_o$  and plotted vs.  $\Delta\psi$ . Note that the rate-limiting step of crossing the energy barrier is identical for  $TEA^+$  and  $H^+$ . ( $H^+$  probably crosses as hydronium ion).

involve reduction of mitochondrial production of reactive oxygen species.

The transport functions and regulation of UCP1–3 have been characterized using recombinant proteins expressed in *E. coli* and reconstituted into liposomes for flux measurements. The purified UCP proteins are qualitatively identical with respect to transport function and regulation. They catalyze electrophoretic flux of protons and alkylsulfonates, and proton flux exhibits an obligatory requirement for fatty acids. Fatty acid-dependent proton transport by UCP1–3 is inhibited by purine nucleotides, including ATP. The mechanism by which UCPs catalyze proton back-flux is somewhat controversial. It is thought that they act as fatty acid anion flippers, causing the outward movement of the fatty acid anion head group across the inner membrane. Once on the outer surface, the fatty acid picks up a proton and then flip-flops rapidly back into the matrix. Thus, UCP does not conduct protons, per se; rather, it enables fatty acids to behave as cycling protonophores, as shown in Figure 7.

#### Fourth Postulate – Ion Carriers and Channels

With astute physiological insight, Mitchell recognized that solving the problem of energy transduction gave rise to another. The high transmembrane electrical potential



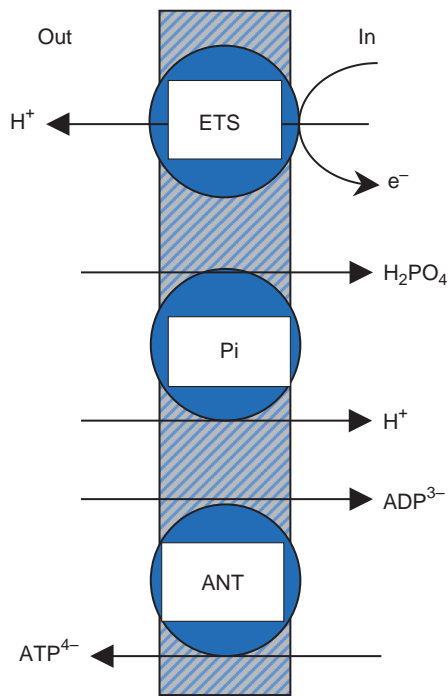
**FIGURE 7** Mitochondrial uncoupling protein (UCP). UCP contains a weak binding site for anions near the center of the membrane. This constitutes an energy well for anions and provides a low-resistance pathway for normally impermeant anions to cross the membrane. The physiological substrates of UCP are anions of free fatty acids (FA). The FA head groups are located at the acylglycerol linkages of the phospholipid bilayer. When the FA is at the surface of UCP, the negative membrane potential drives the head group to the energy well at the center of the membrane. The FA then “flip-flops” and the head group is driven to the opposite surface. Here, it diffuses away from the protein and picks up a proton. The protonated FA (HFA) head group freely diffuses across the membrane, during which the FA flip-flops again, and delivers a proton to the other side. Thus, UCP is a FA anion flipase, and its role is to enable FA to behave as cycling protonophores.

required for ATP synthesis would prevent anions, including the substrates of the tricarboxylic acid cycle, from entering the matrix. It would also promote cation uptake, with consequent osmotic swelling and lysis. Thus, the fourth postulate was born out of physiological necessity.

#### ANION EXCHANGE CARRIERS

Because ATP is synthesized in the matrix, ADP and phosphate must be imported and ATP must be exported across the inner membrane. As shown in Figure 8, nucleotides are exchanged on the ATP/ADP translocase (ANT) in a process involving outward movement of one negative charge. The phosphate carrier catalyzes electroneutral  $P_i/H^+$  symport or  $P_i/OH^-$  antiport, with the net result that it transports phosphoric acid.

The inner membrane also contains a variety of anion exchange carriers, which are designed to deliver substrates to the tricarboxylic acid cycle. The anion exchange carriers catalyze 1:1 electroneutral exchange of anions, and they are arranged in a cascade in which phosphate and malate are key intermediates. In liver



F0040 **FIGURE 8** Mitochondrial transport of inorganic phosphate, ADP, and ATP. The phosphate carrier (Pi) catalyzes electroneutral transport, and the adenine nucleotide translocase (ANT) catalyzes electrophoretic exchange of ADP and ATP. Uptake of Pi and ADP and expulsion of ATP use one electrogenically ejected proton.

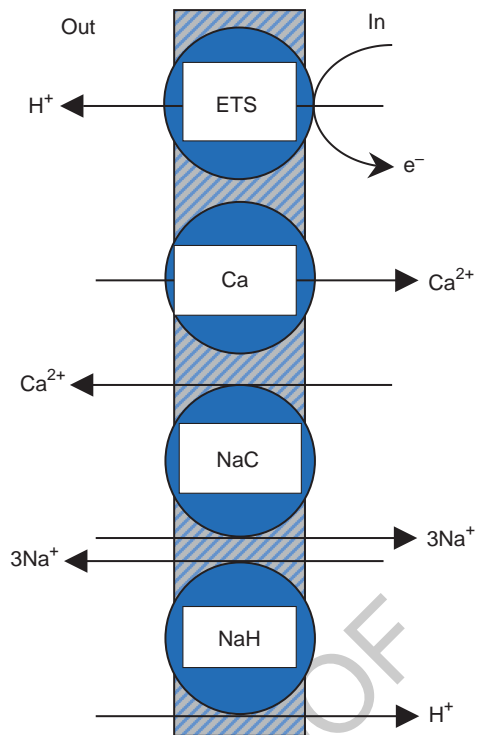
mitochondria, the dicarboxylic acid exchanger catalyzes malate/phosphate exchange, and the tricarboxylic acid exchanger catalyzes malate/citrate exchange. In this way, both di- and tricarboxylic acids are linked to the phosphate carrier. Since the phosphate carrier effectively transports fully protonated phosphate, the net result is that di- and tricarboxylic acids also behave as if they were fully protonated, and they are distributed across the membrane as if they were transported as fully protonated acids:

$$(A^-)_{in}/(A^-)_{out} = 10^{z\Delta pH} \quad (7)$$

where  $z$  is the valence of the acid.

### S0085 THE SODIUM–CALCIUM CYCLE

P0115 The mitochondrial  $Ca^{2+}$  cycle consists of three separate transporters and is diagrammed in Figure 9.  $Ca^{2+}$  is taken up by the  $Ca^{2+}$  channel at the expense of two ejected protons. In spite of the enormous gradient for electrophoretic  $Ca^{2+}$  uptake, free mitochondrial  $[Ca^{2+}]$  is comparable to cytosolic  $[Ca^{2+}]$  *in vivo*. This disequilibrium is maintained in heart mitochondria by an electrophoretic  $Na^+/Ca^{2+}$  antiporter, which exchanges 3  $Na^+$  per  $Ca^{2+}$ . The three  $Na^+$  ions taken up are then ejected by the electroneutral  $Na^+/H^+$



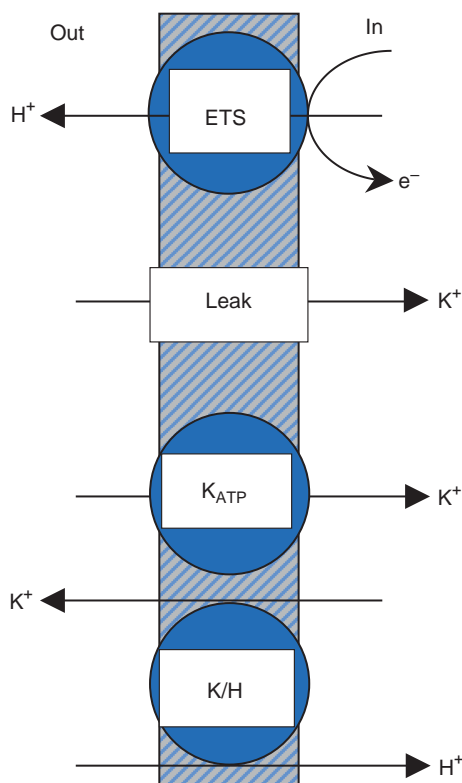
F0045 **FIGURE 9** The mitochondrial  $Ca^{2+}$  cycle.  $Ca^{2+}$  enters the matrix via the electrophoretic  $Ca^{2+}$  channel and is ejected by the electrophoretic  $Na^+/Ca^{2+}$  antiporter (NaC), utilizing three ejected protons per  $Ca^{2+}$  taken up. The  $Na^+$  is then expelled by the electroneutral  $Na^+/H^+$  antiporter (NaH).

antiporter, which holds  $Na^+$  close to equilibrium with the pH gradient.

The physiological role of the mitochondrial  $Ca^{2+}$  cycle is to regulate matrix  $Ca^{2+}$  activity in response to signals from the cytosol. As a second messenger,  $Ca^{2+}$  signals need to increase cellular work. Because increased work requires a higher rate of ATP production, this message must be relayed to the mitochondrial matrix. Intramitochondrial  $Ca^{2+}$  is required to activate the phosphorylase that converts pyruvate dehydrogenase to its active form, and  $\alpha$ -ketoglutarate dehydrogenase is allosterically activated by matrix  $Ca^{2+}$  in the physiological range.

### S0090 THE POTASSIUM CYCLE

P0125 The mitochondrial  $K^+$  cycle consists of electrophoretic  $K^+$  influx and electroneutral  $K^+$  efflux across the inner membrane, as diagrammed in Figure 10. Mitochondria must regulate net  $K^+$  flux to zero in the steady state; otherwise, inward  $K^+$  diffusion would cause the matrix to swell and eventually lyse. This regulation is provided by the  $K^+/H^+$  antiporter, which ejects exactly the amount of  $K^+$  that is taken in. Regulation of the  $K^+/H^+$  antiporter is mediated by reversible binding of



**FIGURE 10** The mitochondrial  $K^+$  cycle. Electrogenic proton ejection drives  $K^+$  uptake by diffusive leak. In addition, the inner membrane contains a  $K_{ATP}$  channel, which is highly regulated by nucleotides, CoA esters, and pharmacological agents. Net  $K^+$  flux is regulated to zero in the steady state. Compensatory  $K^+$  efflux is provided by the electroneutral  $K^+/H^+$  antiporter, which is regulated by matrix  $Mg^{2+}$  and  $H^+$  and is exquisitely sensitive to changes in matrix volume.

$Mg^{2+}$  and  $H^+$  to the  $K^+/H^+$  antiporter on its matrix side. The activity of these ions decreases with uptake of  $K^+$  salts, causing a graded, compensatory activation of  $K^+$  efflux in response to increases in matrix volume. The primary role of the  $K^+/H^+$  antiporter is to provide “volume homeostasis” to mitochondria in order to maintain the vesicular integrity necessary for oxidative phosphorylation.

The mitochondrial  $K_{ATP}$  channel (mito $K_{ATP}$ ) also plays an important role in volume homeostasis. When mito $K_{ATP}$  is open, the added  $K^+$  conductance is thought to compensate for the lower driving force for  $K^+$  influx (lower  $\Delta\psi$ ) in ischemia and in high ATP-consuming states of the cell. Mito $K_{ATP}$  is regulated by a rich variety of metabolic and pharmacological ligands. It is inhibited with high affinity by ATP, long-chain acyl-CoA esters, the antidiabetic sulfonylurea, glyburide, and 5-hydroxydecanoate. The ATP-inhibited channel is opened with high affinity by guanine nucleotides and  $K^+$

channel openers such as cromakalim and diazoxide. There is indirect evidence that mito $K_{ATP}$  is opened *in vivo* by phosphorylation. Mito $K_{ATP}$  has been found to play a pivotal role in protecting heart and brain from ischemic stress. Thus, opening mito $K_{ATP}$ , either with  $K_{ATP}$  channel openers or with endogenous signals, confers significant protection against ischemia-reperfusion injury.

## GLOSSARY

- electrogenic transport** Ion transport (and net charge movement) that requires chemical energy to move an ion across the membrane against its electrochemical potential gradient. Electrogenic transport in mitochondria is limited to proton transport by the electron transport system and by the ATP synthase, when ATP is being hydrolyzed. G0095  
G0135
- electrophoretic transport** Ion transport (and net charge movement) driven by the ion electrochemical potential gradient. In mitochondria, this includes diffusion and transport by ion channels and ionophores such as valinomycin. G0140
- ionophore** A chemical compound that conducts ions across membranes. Examples include valinomycin, CCCP, and nigericin. G0145
- protonophore** An ionophore that conducts protons ( $H^+$  ions) across membranes. Examples include dinitrophenol and CCCP (carbonyl cyanide *m*-chlorophenylhydrazone). G0150

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## Author Queries

- Q1 Refer to hard copy for equation editing.
- Q2 Please update.
- Q3 Please update.
- Q4 Figure 6(A) is not provided. Please check and advise. Thanks.

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