Mitochondrial Electron Transport and Energy Conservation*

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Cells of higher organisms utilize the energy which is made available from oxidation of glucose and other substrates to supply the energy required for cell existence and growth as well as for functions required by the whole organism, such as muscle contraction and nerve transmission (see, for example, ref 1). Energy is made available in a series of controlled oxidation–reduction reactions which synthesize molecules having "high-energy" phosphate bonds. In this context, the term "high energy" is applied to phosphate bonds which, under cellular conditions, have negative Gibbs free energies of hydrolysis greater than 12 kcal/mole. Mechanisms are present in the cell which couple reactions having positive Gibbs free energy changes to the hydrolysis of "high-energy" phosphate bonds in order to permit the cell to perform necessary functions which are otherwise energetically unfavorable. The molecule which serves as the common source of "high-energy" phosphate bonds is adenosine triphosphate (ATP) which is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate.

The metabolic pathways which provide for ATP synthesis may be classified as fermentive (not requiring a source of oxidizing equivalents such as molecular oxygen) and oxidative (requiring a source of oxidizing equivalents). The complete oxidation of 1 molecule of glucose to CO_2 and water using a combination of glycolysis (fermentive pathway) and the tricarboxylic acid cycle (oxidative pathway) yields 2 ATP and 36 ATP, respectively. Glycolysis and the tricarboxylic acid cycle are partitioned within the cell; glycolysis is present in the cytoplasm of the cell while the tricarboxylic acid (Krebs) cycle is localized in a small organelle called the mitochondrion (see, for example, ref 2).

We are concerned primarily with the mitochondrial function commonly called oxidative phosphorylation. The oxidation–reduction components of oxidative phosphorylation are bound to the mitochondrial membrane and transfer the reducing equivalents from the oxidative steps of the tricarboxylic acid to molecular O_2 which is reduced to H_2O (respiratory chain; see ref 3). The transfer of these reducing equivalents is associated with a large negative free-energy change which is conserved by coupling the oxidation–reduction reactions to the synthesis of ATP.

The respiratory chain activity is regulated by the cellular requirement for energy. It functions at a maximal rate when synthesizing ATP under conditions requiring a relatively low negative free energy (high ADP and inorganic phosphate concentrations relative to the ATP concentration), but as the energy required for net ATP synthesis approaches or equals that available in the respiratory chain the respiration is greatly decreased. Under suitable conditions, the direction of the net electron flow within the respiratory chain can be reversed by added ATP, indicating that the complete energy transduction process is reversible. When the number of ATP molecules synthesized per oxygen atom reduced is measured, values of 1, 2, and 3 are obtained depending on the negative free energy change associated with the transfer of the reducing equivalents within the respiratory chain. For reasons which will be more evident later in the paper, energy conservation is considered to occur at three localized regions (sites I, II, and III) of the respiratory chain.

The chemical events which give rise to the phenomenon of oxidative phosphorylation are largely unknown. The respiratory chain contains known oxidation–reduction components as chemically diverse as hemoproteins (at least six cytochromes), quinone (coenzyme Q), iron–sulfur proteins (at least six different species), and flavoproteins. The lack of definitive evidence for chemical intermediates in the transfer of energy from the respiratory chain to ATP has encouraged a proliferation of proposed mechanisms for oxidative phosphorylation. The "chemiosmotic coupling" hypothesis proposed by Mitchell[4] has been the most prominent alternative to the "chemical coupling" hypothesis, and a review by Greville[5] presents a good comparison between them. The basic difference lies in the nature of the primary "high-energy" intermediate. In "chemical coupling" it is a chemical bond specific to the oxidation–reduction species involved. However, in "chemiosmotic cou-

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(1) A. L. Lehninger, "Bioenergetics: A Molecular Basis of Biological Energy Transformations," W. A. Benjamin, Menlo Park, Calif., 1971.
(2) A. L. Lehninger, "The Mitochondria: Molecular Basis of Structure and Function," W. A. Benjamin, New York, N. Y., 1964.
(3) W. W. Wainio, "The Mammalian Mitochondrial Respiratory Chain," Academic Press, New York, N. Y., 1970.
(4) P. Mitchell, *Biol. Rev.*, **41**, 445 (1965).
(5) G. D. Greville in "Current Topics in Bioenergetics," Vol. 3, D. R. Sanadi, Ed., Academic Press, New York, N. Y., 1969, p 1.

pling" no energy is conserved until a pH gradient and/or an electrical potential is generated across the mitochondrial membrane. Chemiosmotic coupling thus postulates the mitochondrial membrane to have the requisite permeability and electrical properties as well as a specific vectorial arrangement of the oxidation–reduction components across the membrane All of these properties must be intact before energy conservation can occur.

In this Account, we present a thermodynamic picture of the mitochondrial respiratory chain and then use this picture as a background for an experimental demonstration that, first, two cytochromes (cytochromes b_T and a_3) are responsible for energy conservation at sites II and III, respectively, and, second, that the primary energy conservation reactions are chemical events which do not require an intact membrane system.

The behavior of oxidation–reduction components is best analyzed according to their oxidation–reduction potentials. Each component may be regarded as one-half of an electrical cell (eq 1) with its electron-donating

$$\text{ox} + ne^- \rightleftharpoons \text{red} \quad (1)$$

tendency described by eq 2. E_h expresses the ten-

$$E_h = E_0' + \frac{RT}{nF} \ln \frac{[\text{ox}]}{[\text{red}]} \quad (2)$$

dency of the couple to accept electrons from a standard hydrogen electrode; E_0' is the characteristic half-reduction potential for the couple; R, T, and F are the gas constant, absolute temperature, and the Faraday constant, respectively. n is the number of electrons transferred when the component is oxidized or reduced. The thermodynamic properties of electron transfer in the general reaction 3 are described in eq 4.

$$A_{red} + B_{ox} \rightleftharpoons A_{ox} + B_{red} \quad (3)$$

$$\Delta E_{A-B} = \Delta E_0'_{A-B} + \frac{RT}{nF} \ln \frac{[A_{ox}][B_{red}]}{[A_{red}][B_{ox}]} \quad (4)$$

ΔE_{A-B} is the potential of the electrical cell formed by the oxidation–reduction couples of A and B and $\Delta E_0'_{A-B}$ is the difference between the characteristic half-cell potentials of the two couples; the remaining symbols have been previously defined. It should be noted that the activity coefficients for the oxidized and reduced forms of a given compound are usually equal[6] and thus may be assumed to be 1 in calculations (they divide out when the ratios are calculated).

A knowledge of the characteristic midpoint potential for the oxidation–reduction components of the respiratory chain is essential to any thermodynamic discussion of the reactions involved because of the relationship that Gibbs free energy change (ΔG) = $-nF\Delta E$. Experimentally, a number of techniques have been used to measure the half-reduction potentials of oxidation–reduction components.[6] These include polarographic techniques, the method of mixtures, and the

(6) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," Waverly Press, Baltimore, Md., 1960.

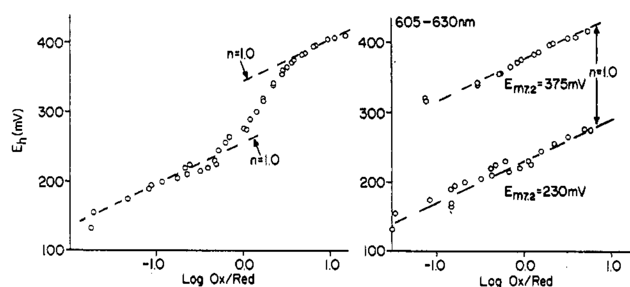


Figure 1. The oxidation-reduction potential dependence of the 605 nm − 630 nm absorbance change of cytochrome oxidase. Pigeon heart mitochondria were suspended at 4 mg of protein/ml in a medium containing 0.22 M mannitol, 0.05 M sucrose, and 50 mM morpholinopropanesulfonate, pH 7.2. Phenazine methosulfate (30 μM) and diaminodurene (50 μM) were added and anaerobiosis was attained by adding aliquots of NADH. After anaerobiosis the absorbance change was titrated oxidatively (ferricyanide addition) and then reductively (NADH addition). The resulting data are plotted with the logarithm of the ratio of the oxidized to reduced form on the abscissa and the measured oxidation–reduction potential relative to a hydrogen electrode on the ordinate. In part A (left) the absorbance change is treated as a single component, while in part B (right) the sigmoid curve of part A is resolved into its two component parts.

potentiometric technique. The last has a much broader applicability and generally greater precision than the other techniques. For this reason, we[7-9] have developed appropriate instrumentation to permit potentiometric techniques to be used to measure the oxidation–reduction midpoint potentials of components in suspensions of biological materials. The methodology has been published in detail. It is sufficient here to note that the method is designed to measure electrometrically the oxidation–reduction potential of an anaerobic sample and either to simultaneously measure by optical techniques the reduction of the component or to anaerobically transfer aliquots of the suspension to electron paramagnetic resonance (epr) sample tubes. The epr sample tubes are then immersed in liquid nitrogen in order to trap the oxidation–reduction state and to permit epr measurements at 77°K.

The Oxidation–Reduction Potentials of the Cytochromes in Intact Mitochondria. We have set out to systematically determine the midpoint potentials and n values of the oxidation–reduction components of the respiratory chain of intact mitochondria. In order to permit analysis, we will present some typical experimental results and then a complete tabulation of the measured values for the components of pigeon heart mitochondria.

In Figure 1A, the oxidation–reduction potential dependence of the reduction of the heme a components in intact pigeon heart mitochondria at pH 7.2 (measured at 605 nm − 630 nm) is presented graphically with the logarithm of the ratio of the oxidized to reduced form on the abscissa and the measured oxidation–

(7) P. L. Dutton, *Biochim. Biophys. Acta*, **226**, 63 (1971).
(8) D. F. Wilson and P. L. Dutton, *Arch. Biochem. Biophys.*, **136**, 583 (1970).
(9) D. F. Wilson and P. L. Dutton, *Biochem. Biophys. Res. Commun.*, **39**, 59 (1970).

reduction potential on the ordinate. As may be seen from eq 2, on such a plot a single component would be expected to appear as a straight line with a slope of 59.3 mV per log unit (n = 1.0) or 29.7 mV per log unit (n = 2.0). The plotted experimental data for the absorbance at 605 nm minus that at 630 nm appears as a sigmoid curve in which the extremities approach the slopes expected for components having n values of 1.0. This indicates that there are two components contributing almost equally to the difference in absorbance between these wavelengths. Mathematical resolution of the curves into two components with n values of 1.0 (Figure 1B) indicates that the two have half-reduction potentials of 375 mV (cytochrome a_3) and 220 mV (cytochrome a). The mathematical separation and identification are justified by the differential reactivity of the two components toward specific ligands such as carbon monoxide and azide.[10,11] In a series of titrations the average half-reduction potentials obtained for cytochromes a_3 and a were 385 and 210 mV, respectively, at pH 7.2. The observed equal contribution of cytochromes a and a_3 to the absorbance at 605 nm minus that at 630 nm is true only when external ligands are not added and this wavelength pair may be used to demonstrate a strong interaction between these cytochromes.[11]

Similar experiments have been made measuring the c cytochromes,[7] the b cytochromes,[7,9] the copper of cytochrome oxidase,[12,13] and the flavoproteins.[14,15] In Figure 2 the measured values of the midpoint potentials of these components at pH 7.2 as determined in our laboratory are presented schematically on a vertical scale of the oxidation–reduction potential as rectangles centered on the midpoint potential and extending from the potential at which the component is 91% reduced. The cytochromes all have n values of 1.0 and the respective midpoint potentials for cytochromes a, a_3, c, c_1, b_K, and b_T are 210, 385, 235, 225, 30, and −30 mV in the uncoupled mitochondria. The midpoint potential (225 mV) for cytochrome c_1 determined *in situ* is in good agreement with the value reported by Green and associates[16] and confirmed by Dutton and associates[7] for the isolated cytochrome c_1; this is the only cytochrome for which the isolated cytochrome has the same value that it has in mitochondria. Previous reports of the midpoint potential of cytochrome a have ranged from near 260 mV for isolated cytochrome oxidase[17,18] to more positive than

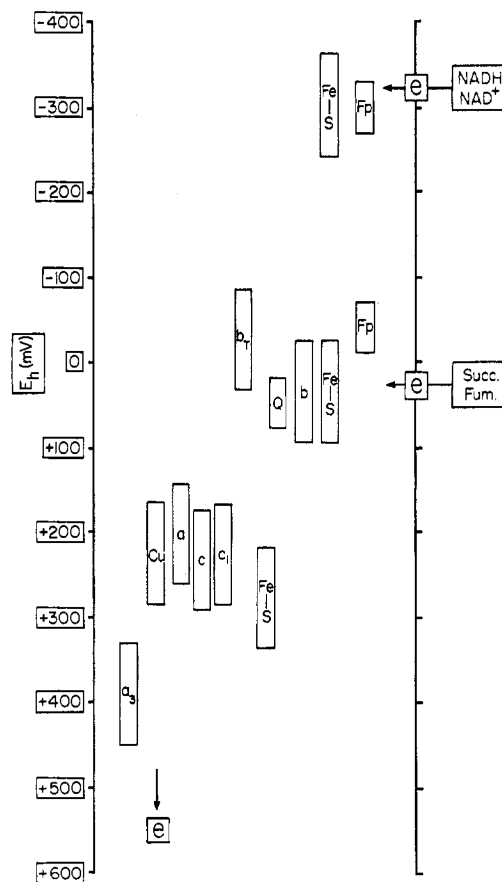


Figure 2. The oxidation–reduction potentials of the respiratory chain components in uncoupled mitochondria. The components are represented by blocks which extend over the potential region over which they become 10–90% oxidized or reduced at pH 7.2.

330 mV in intact mitochondria.[19] Soluble horse heart cytochrome c has a midpoint potential between 250 and 280 mV, depending on the assay medium.[7] When this cytochrome c is bound inside phospholipid vesicles[20] or is in its place in the mitochondrial membrane,[7] the midpoint potential is shifted to 230 and 235 mV, respectively.

The existence of more than one b cytochrome in intact mitochondria has been recognized only recently,[7,9] and the values in the previous literature are reported for beef heart submitochondrial particles assuming a single component[21,22] instead of three components.[7]

The Oxidation–Reduction Potentials of the Copper of Cytochrome Oxidase and of the Flavoproteins of the Respiratory Chain. The copper of cytochrome oxidase is known to be responsible for the 830-nm absorbance band and an electron paramagnetic resonance absorption. The half-reduction potential and n value for the electron paramagnetic resonance absorption of the copper were measured in submitochondrial particles

(10) D. Keilin and E. F. Hartree, *Proc. Roy. Soc., Ser. B*, **125**, 171 (1938).
(11) D. F. Wilson, J. G. Lindsay, and E. S. Brocklehurst, *Biochim. Biophys. Acta*, **256**, 277 (1972).
(12) T. Tsudzuki and D. F. Wilson, *Arch. Biochem. Biophys.*, **145**, 149 (1971).
(13) M. Erecinska, B. Chance, and D. F. Wilson, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **16**, 284 (1971).
(14) M. Erecinska, D. F. Wilson, Y. Mukai, and B. Chance, *Biochem. Biophys. Res. Commun.*, **41**, 386 (1970).
(15) M. Erecinska, D. F. Wilson, Y. Mukai, and T. Ohnishi, (1971), manuscript in preparation.
(16) D. E. Green, J. Jarnefelt, and H. D. Tisdale, *Biochim. Biophys. Acta*, **31**, 34 (1960).
(17) E. G. Ball, *Biochem. Z.*, **295**, 262 (1938).
(18) W. W. Wainio, *J. Biol. Chem.*, **216**, 593 (1955).
(19) A. H. Caswell, *ibid.*, **243**, 5827 (1968).
(20) H. K. Kimelberg and C. P. Lee, *J. Membrane Biol.*, **2**, 252 (1970).
(21) F. A. Holton and J. P. Colpa-Boonstra, *Biochem. J.*, **76**, 179 (1960).
(22) D. Feldman and W. W. Wainio, *J. Biol. Chem.*, **235**, 3635 (1960).

from pigeon heart and were found to be 250 and 1.0 mV, respectively. These values are nearly the same as the values of 240 mV and 1.0 obtained by Erecinska, *et al.*,[13] for the 830-nm absorption band of intact pigeon heart mitochondria. In purified cytochrome oxidase from beef heart, a comparison of the 830-nm absorbance and the epr signal gave half-reduction potentials of 245 mV and n values of 1.0 for both measurements,[12] providing additional evidence that the same copper moiety is responsible for both types of absorption.[23]

The flavoproteins of the mitochondrial respiratory chain have been difficult to measure because of spectral interference by other components and of the multiplicity of the flavin enzymes which are active in the mitochondrial metabolic pathways. In Figure 2, two flavins are given with midpoint potentials of −45 and −300 mV, but these values are mostly speculative. Components have been observed with these values in studies on pigeon heart[14] and yeast mitochondria,[15] but they have not been definitely identified as succinate and NADH dehydrogenases.

The Oxidation–Reduction Potentials of the Mitochondrial Iron–Sulfur Proteins. The number of iron–sulfur proteins associated with the mitochondrial respiratory chain is unknown and their function is a source of considerable speculation. The only reliable method for measuring their oxidation and reduction is epr absorption in frozen samples. These measurements have been used to demonstrate the existence of at least six different species of iron–sulfur proteins which are reduced by substrate.[24,25] The half-reduction potentials of three of these components have been determined to be 280, 30, and −305 mV, and all have n values of 1.0.[26] Urban and Klingenberg[27] have measured the midpoint potential of coenzyme Q in beef heart mitochondria, and this value is presented.

A Thermodynamic Profile of the Respiratory Chain Components and the Energy Conservation Sites. An examination of Figure 3 shows that the midpoint potentials of the components of the respiratory chain form four groups, one at about −300 mV, the second at about 0 mV, the third at about 220 mV, and the fourth at about 400 mV. These divisions roughly correlate with the potential spans utilized for ATP synthesis. The actual oxidation–reduction potential of each component can be estimated from its steady-state per cent reduction, midpoint potential, and n value.

In mitochondria with excess substrate and oxygen but with the respiration decreased by the high ratio of ATP to ADP and inorganic phosphate (state 4), the estimated oxidation–reduction potential of each of

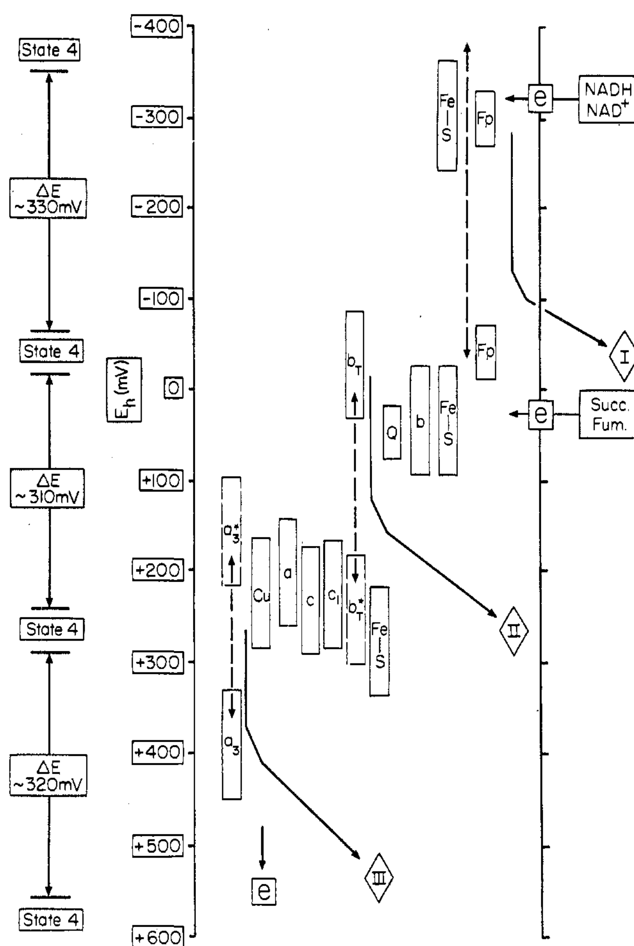


Figure 3. The relationship between the oxidation–reduction potentials of the respiratory chain components and ATP synthesis in coupled mitochondria. The representation of the components is the same as for Figure 2. The dashed blocks for cytochromes b_T and a_3 represent the values obtained when the measurements are carried out in the presence of ATP (no comparable effect of ATP has been observed for the other components). The blocks on the left labeled state 4 are the estimated oxidation–reduction potentials of the designated isopotential group in respiratory state 4 (see text).

the components of the group is very close to that for all other members of the group and the components may thus be regarded as being essentially in equilibrium. For example, cytochromes a, c, and c_1 all have oxidation–reduction potentials near 260 mV. The respiratory chain may be thought of as a series of isopotential groups of components with energy conservation associated with electron transfer between these groups. We have estimated the oxidation–reduction potential for each of the four isopotential groups for mitochondria in state 4 and the values are designated in the boxes on the left side of Figure 3. The approximate potential spans available at the first, second, and third phosphorylation sites (I, II, and III) are 330, 310, and 320 mV, respectively. The values for the first and second sites are readily estimated from direct measurements, but the value for site III makes use of the measured second-order rate constant for the reaction of O_2 with reduced cytochrome a_3 of $8 \times 10^7\ M^{-1}\ \text{sec}^{-1}$,[28] an assumed oxygen concentration of 200 μM, and a

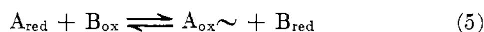
(23) A. Tzagoloff and D. H. MacLennon in "The Biochemistry of Copper," J. Peisach, P. Aisen, and W. E. Blumberg, Ed., Academic Press, New York, N. Y., 1966, p. 253.
(24) T. Ohnishi, T. Asakura, H. Wohlrab, T. Yonetani, and B. Chance, *J. Biol. Chem.*, **245**, 901 (1970).
(25) N. R. Orme-Johnson, W. H. Orme-Johnson, R. E. Hansen, H. Beinert, and Y. Hatefi, *Biochem. Biophys. Res. Commun.*, **44**, 446 (1971).
(26) D. F. Wilson, M. Erecinska, P. L. Dutton, and T. Tsudzuki, *ibid.*, **41**, 1273 (1970).
(27) P. F. Urban and M. Klingenberg, *Eur. J. Biochem.*, **9**, 519 (1969).

measured half-time for the oxidation of cytochrome a by cytochrome a_3 of 6 msec. These values permit us to estimate that in state 4 the cytochrome a_3 is only 0.1% reduced.

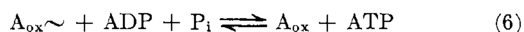
When electrons are transferred from the pyridine nucleotide linked substrate to cytochrome a_3 the potential change (ΔE) is approximately 1 V (960 mV). The Gibbs free energy available for ATP synthesis on transfer of two electrons through this potential span may be calculated to be approximately −45 kcal. When this available energy is distributed to three phosphorylation sites, each individual site can supply approximately −15 kcal. Cockrell and associates[29] have measured the maximum phosphate potential which could be formed by mitochondria under state four conditions and found it to be 15.6 kcal. These values are equal within the limits of the available data as would be expected from the reversibility of the reactions.

In Figure 3 there are two values given for the half-reduction potentials of cytochrome b_T and a_3. We have observed that when the potential measurements are made at pH 7.2 in the presence of excess ATP,[8,9,30] the half-reduction potential of the cytochrome b_T is +245 mV (as compared to −30 mV in uncoupled mitochondria) while the half-reduction potential of cytochrome a_3 is approximately +160 mV (as compared to +385 mV in uncoupled mitochondria). No comparable ATP dependence has been observed for any of the other components which have been measured.[30a]

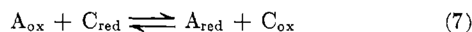
The Nature of the Energy Transducing Reaction. The energy transduction reaction itself may profitably be evaluated in thermodynamic terms since such an evaluation predicted the observed energy dependence of the half-reduction potentials[8,9] as well as other experimentally accessible conclusions. It is important to note that the oxidation–reduction reactions involve electron transfer while ATP synthesis from ADP and inorganic phosphate does not.[31,32] The minimum equations containing the required information for an energy conservation site are given in eq 5–7. The



$$A_{red} + B_{ox} \rightleftharpoons A_{ox}\sim + B_{red} \quad (5)$$



$$A_{ox}\sim + ADP + P_i \rightleftharpoons A_{ox} + ATP \quad (6)$$

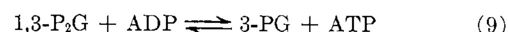


$$A_{ox} + C_{red} \rightleftharpoons A_{red} + C_{ox} \quad (7)$$

energy transducer is designated a two-electron carrier, A, and the other electron carriers are designated B and C in order to avoid mechanistic arguments which are special cases and must conform to the general evaluation.

The equations describe a mechanism in which ATP is synthesized by electron flow from the low potential pool ($C_{red} \rightarrow C_{ox}$) to the high potential pool ($B_{ox} \rightarrow B_{red}$). This directional electron flow serves to maintain the ratio of the activities of the $A_{ox}\sim$ to the A_{ox} such that the free energy of interconversion of the two species is equal to, or greater than, the energy required to synthesize ATP. This mechanism is quite general and is also an accurate representation of the ATP synthesis catalyzed by glyceraldehyde-3-phosphate (G-3-P) dehydrogenase (eq 8 and 9), where 1,3-P_2G

$$\text{G-3-P} + NAD^+ + P_i \rightleftharpoons 1,3\text{-}P_2G + NADH \quad (8)$$



$$1,3\text{-}P_2G + ADP \rightleftharpoons \text{3-PG} + ATP \quad (9)$$

represents 1,3-diphosphoglycerate and 3-PG represents 3-phosphoglycerate), except that the glyceraldehyde 3-phosphate mechanism does not include a reaction equivalent to eq 7; thus net ATP synthesis requires a net consumption of glyceraldehyde 3-phosphate. The form of the equations and the equilibrium behavior of the two systems (eq 5–7 and eq 8 and 9) are very similar. In each case the active component has one reduced form (A_{red} and G-3-P) and two oxidized forms $A_{ox}\sim$ and A_{ox}; 1,3-P_2G and 3-PG). At equilibrium the ratio of the concentrations of the two oxidized forms is dependent on the phosphate potential. As a result, at a high phosphate potential the dominant oxidized forms are $A_{ox}\sim$ and 1,3-P_2G, respectively, while at low phosphate potential the dominant oxidized forms are A_{ox} and 3-PG, respectively. It is not surprising therefore that the oxidation–reduction potential required to give a 50% reduction (half-reduction potential) is dependent on the phosphate potential for both reaction mechanisms. A complete analysis of the reaction mechanisms and a consideration of the kinetic requirements of oxidative phosphorylation have been made and the properties of the energy-transducing reaction may therefore be summarized as follows. (a) The primary energy conservation event is the formation of a new chemical derivative of the energy-transducing electron transport component. (This new species is part of an oxidation–reduction couple having a different half-reduction potential from that of the original component.) (b) The experimentally measured half-reduction potential of the energy-transducing element is dependent on the phosphate potential. (c) This measured half-reduction potential changes by more than 240 mV and is limited at one extreme by the value for the deenergized system and at the other extreme by the value for infinite phosphate potential. (d) A measured half-reduction potential which has a more negative value with high phosphate potential implies a "high-energy" reduced form, while a more positive value implies a "high-energy" oxidized form.

The only components for which we have been able to demonstrate an energy dependence of the measured oxidation–reduction midpoint potentials are cytochrome b_T and a_3;[8,9] this appears to establish these

(28) B. Chance, M. Erecinska, and E. M. Chance in "Oxidases and Related Redox Systems II," T. E. King, H. S. Mason, and M. Morrison, Ed., University Park Press, Baltimore, Md.

(29) R. S. Cockrell, E. J. Harris, and B. C. Pressman, *Biochemistry*, **5**, 2326 (1969).

(30) D. F. Wilson, J. S. Leigh, Jr., J. G. Lindsay, and P. L. Dutton, in ref 28.

(30a) NOTE ADDED IN PROOF. Experiments completed after this paper was submitted show that on addition of ATP, the half-reduction potential of cytochrome a changes from +210 to +260 mV (J. G. Lindsay and D. F. Wilson, unpublished results) and that of cytochrome c_1 changes from +225 to +175 mV (P. L. Dutton and J. G. Lindsay, unpublished results).

(31) E. C. Slater, *Nature (London)*, **172**, 975 (1953).

(32) B. Chance and G. R. Williams, *Advan. Enzymol.*, **17**, 65 (1956).

two cytochromes as the oxidation–reduction components most likely to be directly involved in energy transduction at phosphorylation sites II and III, respectively.

On the Identification of the High-Energy Forms of the Energy-Transducing Components. Although the measured energy dependence of the half-reduction potentials of cytochromes b_T and a_3 implicates them in energy transduction, our analysis now calls specifically for the energy-dependent interconversion of two oxidized forms of cytochrome b_T and of two reduced forms of cytochrome a_3. We have undertaken therefore a systematic search for energy-dependent spectral shifts in the respiratory chain components. The first definitive evidence for the spectral shift of an oxidized cytochrome[33] is shown in Figure 4.

The Spectral Changes Induced by the Addition of ATP to Pigeon Heart Mitochondria in Which the Cytochromes are Highly Oxidized. The addition of ATP to a suspension of pigeon heart mitochrondria in which the cytochromes are highly oxidized induces a spectral change characterized by maxima near 580 and 434 nm and minima at 414, 480, and 655 nm (Figure 4). This spectral change is prevented by the presence of either oligomycin or uncoupler. The sensitivity to these compounds indicates that the phenomenon is energy dependent. The spectrum presented in Figure 4B was actually obtained by treating the mitochondria in the reference cuvette with an excess of oligomycin and then adding ATP to both the measure and the reference cuvettes.

The Identification of the Change as Belonging to an Oxidized Cytochrome. Although pigeon heart mitochondria have a very low content of endogeneous substrate, it remains possible that the observed spectral changes are caused by an energy-dependent reduction of an as yet unknown component of the respiratory chain. Two lines of evidence argue against this possibility. (1) The ATP-induced spectral change is found to be undiminished in size when the mitochondria are suspended in a medium saturated with 100% oxygen gas and supplemented with 1.5 mM potassium ferricyanide in order to maintain highly oxidizing conditions. As may be seen from the spectra, ATP causes only a negligible reduction of any of the known components of the mitochondrial respiratory chain. (2) In separate experiments performed under anaerobic conditions in the presence of ATP, the absorbance appears on addition of oxidant and disappears on the addition of reductant. Attempts to determine the half-reduction potential of the component responsible for the absorbance change indicate that the value is approximately +235 mV at pH 7.6 and that the n value is 1.0.

The spectral change is truly "energy dependent" in the sense that the measured phosphate potential ([ATP]/([ADP][P$_i$])) required for one-half of the change is approximately 10^3 M^{-1}. This value corresponds to a Gibbs free energy change of 12–13 kcal. The ATPase activity present in the mitochondria causes an overestimation of this value, but the overestimation is unlikely to be greater than 1–2 kcal.

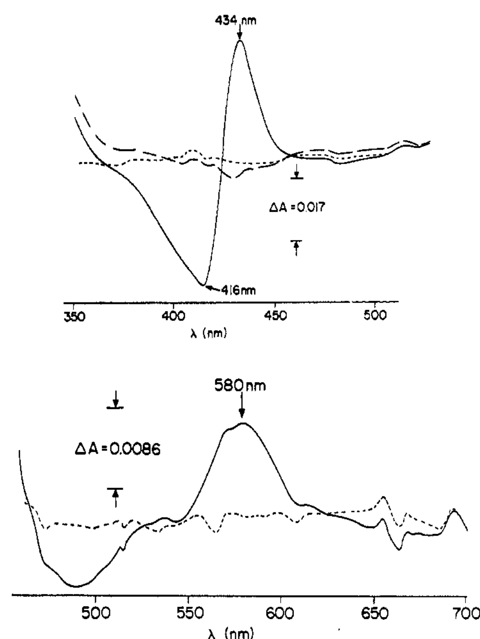


Figure 4. The spectral change induced by adding ATP to pigeon heart mitochondria in which the cytochromes are highly oxidized. The pigeon heart mitochondria were suspended in an oxygen-saturated medium containing 0.225 M mannitol, 0.075 M sucrose, 1.5 mM potassium ferricyanide, and 0.05 M morpholinopropanesulfonate, pH 7.2. Spectra were obtained using an Aminco-Chance dual wavelength/split-beam recording spectrophotometer and a 1-cm light path. The measuring light had a bandwidth at half-height of 4 nm. (A, top) A mitochondrial suspension containing 1.5 mg of protein/ml was used to fill both cuvettes and a zero difference spectrum (- - -) was obtained. The mitochondrial suspension in the measure cuvette was then treated with 4 mM ATP. The spectrum of the resulting difference in absorbance was then measured (———) and then 0.3 μM 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide was added to the measure cuvette to uncouple the mitochondria and the spectrum again measured (– –). (B, bottom) A mitochondrial suspension containing 5 mg of protein/ml was used to fill both cuvettes and a zero difference spectrum (- - -) was obtained. The suspension in the reference cuvette was then treated with 0.5 μg of oligomycin/mg of protein and 4 mM ATP was added to both cuvettes. The spectrum of the resulting difference in absorbance was then measured (——).

On the Possible Identification of the Component Undergoing the Energy-Dependent Change. The identification of the component is not complete, but present evidence suggests that it is a hemoprotein associated with energy conservation site III.[33] The spectrum is consistent with the spectral change which occurs when an oxidized hemoprotein changes from a high-spin form (low phosphate potential) to a low-spin form (high phosphate potential).[34,35] This transition appears to be insensitive to antimycin A but is blocked by pretreatment with cyanide.

The Effect of ATP Addition to Mitochondria in Which the Cytochromes Are Highly Reduced. The

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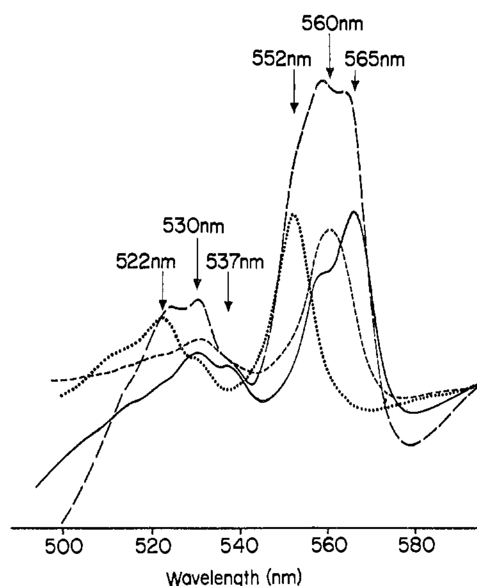

Figure 5. The difference between the spectra of the reduced and oxidized cytochromes of the succinate–cytochrome c reductase. A purified succinate–cytochrome c reductase preparation was diluted to 3.5 M cytochrome c_1 with a 50 mM morpholinopropanesulfonate buffer, pH 7.2. (····) The sample in the reference cuvette was untreated while the sample in the measure cuvette was treated with 100 μM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and 3 mM sodium ascorbate. (- - -) The sample in the reference cuvette was treated with 100 μM TMPD and 3 mM sodium ascorbate while the sample in the measure cuvette was treated with the TMPD, ascorbate, and succinate (6 mM) and fumarate (1 mM) mixture. (——) The sample in the reference cuvette was treated with sodium hydrosulfite to obtain complete reduction. (— —) The sample in the reference cuvette was untreated while the sample in the measure cuvette was treated with sodium hydrosulfite.

addition of ATP to mitochondria in which the cytochromes are maintained highly reduced by a combination of anaerobiosis, substrate, and the appropriate oxidation–reduction mediators provides evidence for an energy-dependent interconversion of two species of reduced cytochrome a_3. The α absorption maximum shifts slightly (less than 2 nm) to longer wavelength when ATP is added, as evidenced by the appearance in the difference spectrum of a trough near 600 nm and a maximum near 616 nm. This absorbance change is not due to oxidation or reduction but is an energy-dependent absorbance change in a ferrocytochrome by the same criteria as previously applied to the ferricytochrome absorbance change. The identification of reduced cytochrome a_3 (as opposed to cytochrome a) as the form undergoing the spectral shift is inferred from the half-reduction potential measurements. Our current uncertainty as to the spectral properties of cytochromes a and a_3 and the strong heme–heme interaction between the cytochromes[11] make it very difficult to unequivocally assign the spectral shift to cytochrome a_3.

The energy-dependent spectral changes thus far observed provide additional evidence for a direct involvement of the cytochromes in energy conservation but as yet they are insufficiently understood for more detailed interpretation. Model systems for oxidative phosphorylation such as those devised by Wang[36,37] may be of great value in understanding the role of the cytochromes in this process.

Reports by Slater and coworkers[38,39] of an energy-dependent "red shift" in the α plane maximum of reduced cytochrome b are based on experimental data which are readily explained by the existence in the respiratory chain of two b cytochromes, one of which (b_T) has an α maximum at 565 nm and an energy-dependent half-reduction potential while the other (b_K) has an α maximum at 561 nm and an energy-independent half-reduction potential. There is as yet no measured effect of the mitochondrial energy state on the spectra of cytochromes b_K or b_T.[40,41]

On the Absence of a Requirement for a Membrane for Energy Conservation. The study of energy coupling mechanisms in electron transport has proven very difficult, partly because the electron transport chain is membrane bound and has resisted efforts for fractionation and purification with retention of a measureable coupling activity. The energy-dependent midpoint potential changes in cytochromes b_T and a_3 are properties of components of the respiratory chain which can, in theory, be used to measure energy conservation associated with respiration in the absence of the additional enzymes required for the conventional assays for energy conservation (ATP synthesis, energy-linked transhydrogenase, etc.)

The succinate–cytochrome c reductase is an ideal preparation in which to test for energy conservation in an isolated fragment of the respiratory chain.[42,43] It is prepared by detergent solubilization of the mitochondrial membrane and is free of the cytochromes a, a_3, and c. In addition, the succinate–fumarate couple may be used as a convenient source of electrons at a defined oxidation–reduction potential.

The succinate–cytochrome c reductase contains three spectrally distinct cytochromes (Figure 5). The α absorption maximum of the c cytochrome is at 553 nm (cytochrome c_1) while the two b cytochromes have α maxima at 561 nm (cytochrome b_K) and at 565 nm with a shoulder at 558 nm (cytochrome b_T). These spectra are the same as were reported originally for these cytochromes in pigeon heart mitochondria by Sato, *et al.*[40,41]

When a preparation of succinate–cytochrome c reductase is supplemented with 1.2 mM succinate and the reduction of the b cytochromes monitored at 561 nm minus 575 nm, the b cytochromes can be observed

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to rapidly attain a steady-state level of reduction (Figure 6). The addition of 4 μM phenazine ethosulfate (PES) initiates an oxygen consumption because it is readily reduced by succinate and reoxidized by molecular oxygen. The PES concentration is extremely low, and some minutes are required before the oxygen in the medium is exhausted and further reduction of the b cytochromes is observed. Because the reaction chamber is completely sealed and the gas phase maintained oxygen free with a continuous stream of ultrapure argon (<1.0 ppm of O_2), the sample is strictly anaerobic. The addition of 0.5 μg of antimycin A and 10 mM fumarate establishes a new, more oxidized level of the b cytochromes which corresponds to an oxidation–reduction potential of approximately +65 mV as estimated both from the fumarate–succinate ratio and by potentiometric measurement of the PES. As shown in trace A, ferricyanide addition causes an absorbance change which indicates a large reduction of the b cytochrome (see also ref 44). This absorbance change is reversed on complete conversion of the ferricyanide to ferrocyanide (largely through the PES bypass of the antimycin inhibitory site). A reduction of the b cytochrome on addition of ferricyanide would normally be unexpected because ferricyanide is a strong oxidant (half-reduction potential is +420 mV). In these preparations, however, the ferricyanide preferentially oxidizes cytochrome c_1 and it has the function of activating electron transport through the electron-transport system from cytochrome b to cytochrome c_1 (through energy conservation site II). Part of the proof that this activation of electron transport is indeed responsible for the absorbance change is shown in trace B in which an experiment is carried out but the electron transport is activated by H_2O_2 in the presence of cytochrome c and cytochrome c peroxidase. The same effect is obtained by oxygen addition if cytochrome c and cytochrome oxidase are added.

The spectrum of the ferricyanide induced absorbance change[35,38] clearly shows the reduction of a b cytochrome having an α maximum at 565 nm and a shoulder near 560 nm and an oxidation of cytochrome c_1. The spectrum of the b cytochrome is characteristic of cytochrome b_T.[40,41] The complete reduction of the cytochrome b_T (half-reduction potential = −30 mV in the unenergized form) by the succinate–fumarate couple at +65 mV is an energy-requiring process. The experimental data indicate that the half-reduction potential of cytochrome b_T becomes greater than +125 mV. A similar shift in intact mitochondria is obtained by a phosphate potential of greater than $10^2\ M^{-1}$ (a ΔG for ATP hydrolysis of greater than 11.6 kcal).[45]

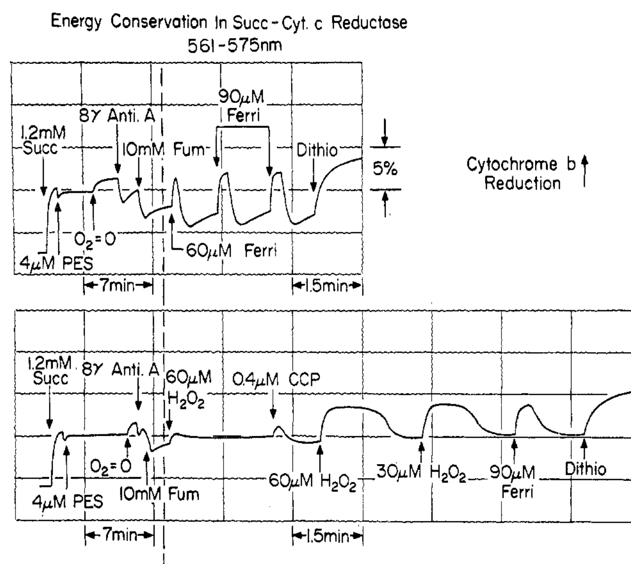

Figure 6. Energy conservation in the purified succinate–cytochrome c reductase. The succinate–cytochrome c reductase was suspended in a medium containing 50 mM morpholinopropanesulfonate as a buffer at pH 7.2. The final cytochrome c_1 concentration was approximately 1.3 μM and additions were made at the indicated times. Abbreviations used are succ (succinate), PES (phenazine ethosulfate), γ (micrograms), Anti. A (antimycin A), Fum (fumarate), CCP (cytochrome c peroxidase), Ferri (ferricyanide), and dithio (sodium hydrosulfite) (taken from ref 43; reproduced with the permission of Academic Press). Trace A, top; trace B, bottom.

The observation that the primary energy-coupling reactions are still present and measurable in purified fragments of the respiratory chain is most important for future work. The energy-coupling reactions have thus been removed from the vitalistic realm of membrane biochemistry and may now be studied by the techniques which have been used so successfully to study soluble enzyme mechanisms.

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