Journal of Bioenergetics and Biomembranes, Vol. 12, Nos. 5/6, 1980

The Effect of Membrane Potential on the Redox State of Cytochrome b_{561} in Antimycin-Inhibited Submitochondrial Particles[1]

Asher Gopher and Menachem Gutman

Department of Biochemistry
G.S. Wise Faculty of Life Science
Tel-Aviv University, Tel-Aviv, Israel

Received February 4, 1980

Abstract

The oxidation of cytochrome b_{561} by ATP was measured in submitochondrial particles inhibited by antimycin. The redox potential of the bulk (*M* phase) was controlled by the ratio of fumarate:succinate, and the oxidation of cytochrome *b* was calculated and expressed as a change in redox potential (E_h) measured in millivolts. The oxidation of cytochrome b_{561} is an energy-driven reaction affected only by the $\Delta\psi$ component of the proton motive force. The oxidation (measured in millivolts) is a function of the phosphate potential, reaching a maximal value of 40 mV at $\Delta G'_{ATP} < -12$ kcal/mole. The maximal measured value of ATP-dependent $\Delta\psi$ was 100 mV. Thus only a fraction of the membrane potential effects the redox state of cytochrome b_{561}. In contrast to the ATP-induced oxidation of cytochrome b_{561}, cytochrome b_{566} is in redox equilibrium with fumarate succinate either in the presence or in the absence of ATP. The selective oxidation of b_{561} is explained within the term of the *Q* cycle as a reflection of $\Delta\psi$ on the electron electrochemical potential. The positive electric potential of the *C* phase causes cytochrome b_{566} to act as oxidant with respect to cytochrome b_{561}. In the presence of antimycin cytochrome b_{561} cannot equilibrate with the quinone and undergoes oxidation, while cytochrome b_{566} reequilibrates with the quinone and thus regains redox equilibrium with the fumarate succinate redox buffer.

Introduction

The oxidation of cytochrome *b* by ATP in antimycin-inhibited mitochondria is known for long [1–3]. It has been demonstrated that the oxidized species is

[1]Abbreviations used: ETP_H, phosphorylating submitochondrial particles; TMPD, $N_1N_1N'N'$-tetramethyl-*p*-phenylenediamine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; Mes, 2-(*N*-morpholino) ethanesulfonic acid.

0145-479X/80/1200-0349$03.00/0

b_{561} [2, 3] and that oxidation calls for the presence of antimycin. While the experimental observations are clear and accurate, the mechanism leading to the oxidation is still ambiguous. According to Slater and Lee [3] the oxidant is an unknown species and two coupling sites were proposed in the dehydrogenase-quinone–cytochrome *b* junction. The interpretation given by Flatmark and Pedersen [2] utilizes the term "transducing carrier." This nomenclature is not fully compatible with the present role given to the proton motive force as the intermediate in coupling reactions. Thus though Wikstrom [4] suggested that $\Delta\psi$ might shift the redox potential of the cytochromes, he still classified this oxidation among the "less well-known" reactions of cytochrome *b*.

The Q cycle describes cytochrome b_{561} as a carrier which participate, together with the dehydrogenases, in the reduction of the quinone on the *M* side of the mitochondrial membrane. The redox reaction of cytochrome b_{561} with the quinone is the antimycin-sensitive reaction of the electron transport chain. According to Mitchell [5] and Wikstrom [4] cytochrome b_{561} can be reduced by cytochrome b_{566}, which is located on the *C* side of the membrane. Thus considering the thermodynamically unfavorable reduction of cytochrome b_{566} by b_{561}, cytochrome b_{561} is unique in its ability to be reduced by carriers located on the *M* side or the *C* side of the membrane. In the absence of antimycin and in the presence of KCN and ascorbate plus TMPD (in order to keep *c* and *a* type cytochromes in a fully reduced state) b_{561} is reduced by the dehydrogenases and attains a redox equilibrium with the *M* phase. In the presence of antimycin, the equilibrium of cytochrome b_{561} with the *M* phase redox couple will be mediated by cytochrome b_{566}, which is located on the *C* side of the membrane [4, 5]. Indeed evidence for such a dual pathway for reduction of the *b* type cytochromes was observed by Trumpower in his oxidation-factor-depleted preparation [6].

As in the presence of antimycin, cytochrome b_{561} equilibriates with the substrate (on the *M* side) only via cytochrome b_{566} (located on the *C* side), and its redox state will be altered whenever the electrochemical potential of the protons of the two phases are not the same. Acidification of the *C* phase (due to ATPase-linked proton translocation) will increase the redox potential of cytochrome b_{566} [7], which will act as oxidant with respect to cytochrome b_{561}. Similarly, according to Walz [8], $\Delta\psi$ (positive on *C* side) will alter the electron electrochemical potential ($\tilde{\mu}_e$), causing carriers located on the *C* side of the membrane to behave as oxidants with respect to carriers on the *M* side of the membrane. The effect of either mechanism will be an oxidation of cytochrome b_{561}. Consequently the interpretation of the energy-dependent oxidation of cytochrome b_{561} calls for a possible distinction between ΔpH and $\Delta\psi$ as the driving force.

The previous studies on oxidation of cytochrome b_{561} were carried out

under conditions where the redox potential of the substrate was not defined. Similarly the oxidation of b_{561} was not expressed in millivolts. Consequently under such conditions the extent of oxidation is not simply related to the magnitude of the driving force. In order to improve our ability to interpret the results, we carried out our experiments in a titrative mode. This technique enabled us to relate the redox potential measured for cytochromes b_{561} and b_{566} with the redox potential of the substrate. As will be documented below, such quantitative approach led to unambiguous assignment of $\Delta\psi$ as the driving force for oxidation of cytochrome b_{561}.

Materials and Methods

Methods

Beef heart mitochondria were isolated according to Ringler et al. [9]. Submitochondrial particles from these mitochondria were prepared as described by Hansen and Smith [10]. The ETP_H were routinely suspended in 180 mM sucrose, 50 mM Tris-acetate, and 5 mM $MgSO_4$ (sucrose-Tris buffer). Spectra and steady-state absorbance were measured using an Aminco DW 2 spectrophotometer.

The value of $\Delta\psi$ was measured either by the safranine method [11] or by equilibrium dialysis [12] using $K^{14}CNS$, assuming an internal volume of 1 μl/mg protein [13]; ΔpH was measured according to Rottenberg et al. using 9-aminoacridine [14]. Oxidase activity was measured polarographically (Clark electrode).

Data Evaluation

The redox state of cytochromes b_{561} and b_{566} were calculated from changes in the absorbance at the wavelength pairs 561–575 and 566–575 nm. The absorbancies of these wavelengths were analyzed as described by Wilson and Erecinska [15], ascribing the absorbance at each wavelength to the contribution of the two cytochromes. The relative contribution of each cytochrome to the absorbance is given by the equations

$$\Delta A_{561-575} = 0.75(b_{561}) + 0.25(b_{566})$$

$$\Delta A_{566-575} = 0.45(b_{561}) + 0.55(b_{566})$$

The parameters used in the above equations were measured according to Wilson and Erecinska [15], and slightly differ from their values (see Discussion).

The maximal oxidation and reduction level of each *b* type cytochrome was taken as that measured either aerobically in the absence of substrate plus

1 μM FCCP (oxidized) or when reduced by $Na_2S_2O_4$ plus antimycin, respectively. It should be emphasized that our ETP_H preparations are devoid of endogenase substrate as verified by measuring the effect of KCN on the absorbance spectrum.

The redox potential of the fumarate succinate couple was calculated according to the following equation [16]:

$$E_h = 24 + 30 \log ([\text{Fumarate}]/[\text{Succinate}]) - 60(\text{pH} - 7.0)$$

The redox potential of the *b* type cytochrome was calculated from their redox state (b_{ox}/b_{red}) and the respective mid-potentials (E_m). The mid-potentials of the *b* type cytochrome used in our calculations were: b_{561} (+ antimycin) = 70 mV; b_{561} = 50 mV; b_{566} (+ antimycin) = 30 mV; and b_{566} = −62 mV. These values were determined by redox titration at pH 7.4 except that of b_{566} = −62 mV, which is taken from Erecinska and Wilson [17] and corrected for the change in pH.

In all cases care was taken to ensure that the measured absorbance reached its equilibrium level, either by repeated scanning or by following the absorbance changes at constant wavelength until the reading was constant with time.

Results

Oxidation of Cytochrome b *by ATP in Submitochondrial Particles*

Inside-out submitochondrial particles expose the succinate binding site to the bulk of the solution. Thus in contrast with intact mitochondria, the limited permeability of fumarate across the membrane does not hamper our ability to impose a known redox potential by this redox couple. In the following experiments we used this system in order to investigate the mechanism of oxidation of the *b* type cytochromes by ATP. Furthermore in order to prevent redox changes in the *c* and *a* type cytochromes, KCN, ascorbate, and TMPD were always present in the reaction mixture.

In accordance with Flatmark and Pedersen [2] we observed an enhanced reduction of the *b* type cytochromes upon addition of ATP to ETP_H pretreated with ascorbate, TMPD, KCN, and fumarate plus succinate at a molar ratio of 10 (not shown). When antimycin was added prior to ATP, addition of ATP caused an oxidation of cytochrome *b*, having a maximum at 562.5 nm (Fig. 1 and inset). While such measurements have been reported before, the analysis of the absorption changes for the relative contribution of cytochromes b_{566} and b_{561} was not carried out. As shown in Fig. 2A, the absorbance of our experimental system at 561 nm is highly dependent on antimycin concentration. If ATP is present, the absorbance at this wave-

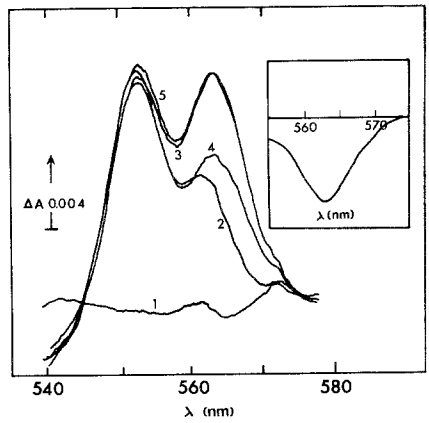


Fig. 1. The effect of ATP on the redox state of the *b* cytochromes in ETP_H in the presence of antimycin A. Dual-wavelength spectral scanning (isosbestic reference point at 575 nm). ETP_H (1.7 mg/ml) were suspended in 0.18 M sucrose, 50 mM Tris-acetate, pH 7.4, and 5 mM $MgSO_4$ (30°C) and the spectra were measured after the following sequential additions: (1) oxidized baseline; (2) addition of 2 mM KCN, 66 μM TMPD, 4 mM ascorbate, 10 mM fumarate, and 1 mM succinate; (3) antimycin A (2 nmoles/mg protein); (4) ATP (1 mM); (5) FCCP (1 μM). Insert—difference spectra showing the effect of ATP obtained by subtracting line 4 from line 3.

length is nearly constant and independent of antimycin concentration. This observation is more informative once the redox state of each *b* type of cytochrome is calculated according to its absorption at the two wavelengths 566 and 561 nm [15]. The results of these calculations are presented in Fig. 2B and C.

Titration of cytochromes b_{561} or b_{566} with increasing concentrations of antimycin shift their redox state by 40 mV and 60 mV respectively in a sigmoidal curve. The inflection point and end point of this curve are identical with those measured for inhibition of succinoxidase activity by antimycin (not shown).

The effect of antimycin on the redox states of cytochromes b_{566} and b_{561} (in the absence of ATP) is readily explained by the known effect of antimycin on the redox mid-potential (E_m) of cytochrome b_{561} and b_{566} [17]. If we calculate, for the extreme points (in the absence of or in saturation by antimycin), the potential of cytochrome *b* [$E_h = E_m + 60 \log (b_{ox}/b_{red})$] a constant value is obtained for cytochromes b_{561} and b_{566}. This value is practically identical with the applied potential of the fumarate succinate couples (see Fig. 9).

Repeating these antimycin titrations in the presence of ATP alters the response of the cytochromes. The effect of ATP can be studied now as a function of the degree of inhibition of the electron transport system by antimycin. At low concentrations of antimycin the redox state of b_{561} is unaffected by ATP. Once the inhibitor concentrations are high enough to block electron transport, cytochrome b_{561}, in the presence of ATP, assumes a redox state some 40 mV more positive than those measured in the absence of ATP. The response of b_{566} to ATP is more ambiguous. In the presence of saturating concentrations of antimycin the redox state of b_{566} is practically that of the fumarate succinate couple whether ATP is present or not (see Fig.

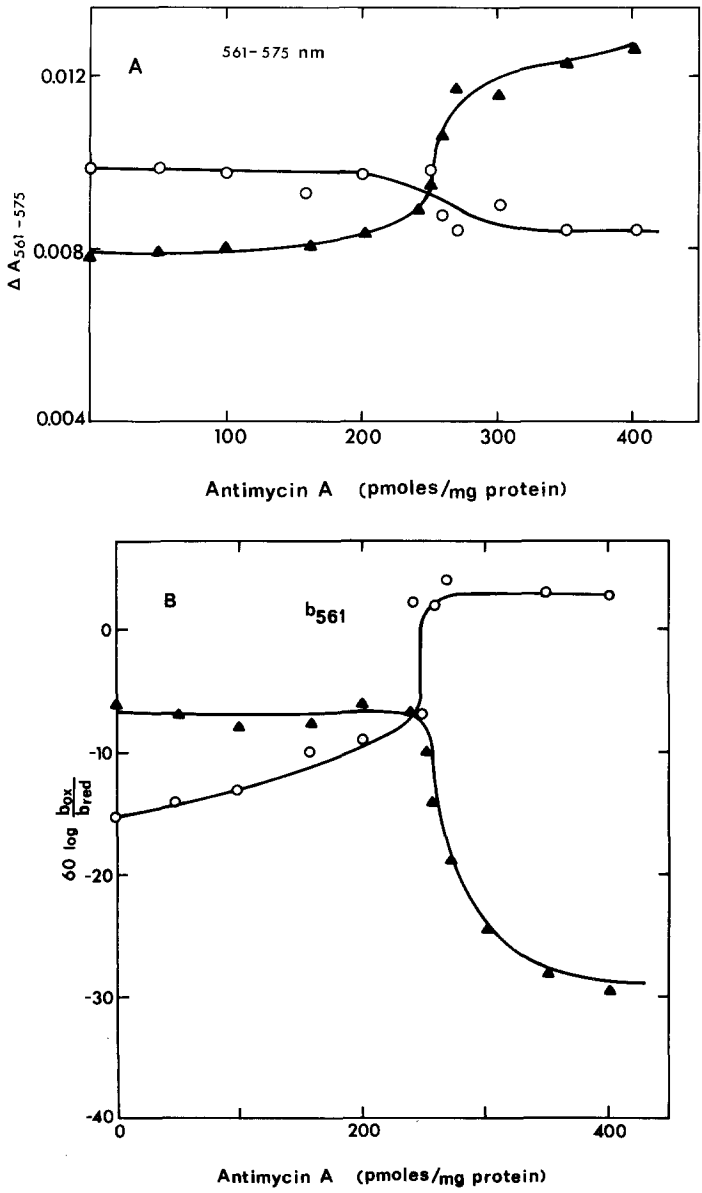


Fig. 2. The effect of antimycin A concentration on the response of cytochromes b_{561} and b_{566} to ATP. ETP_H (1.5 mg/ml) were suspended in sucrose-Tris buffer and the cytochrome *b* was reduced by addition of 2 mM KCN, 4 mM ascorbate, 66 μM TMPP, 10 mM fumarate, and 1 mM succinate followed by successive additions of antimycin and 1 mM ATP. The spectra between 540–610 nm using 575 nm as reference wavelength were recorded. (A) The absorbance change at 561–575 nm before (▲) and after (○) the addition of ATP. The redox states of cytochromes b_{561} and b_{566} before (▲) and after (○) the addition of ATP were calculated as described in Methods for cytochrome b_{561} (B) and cytochrome b_{566} (C).

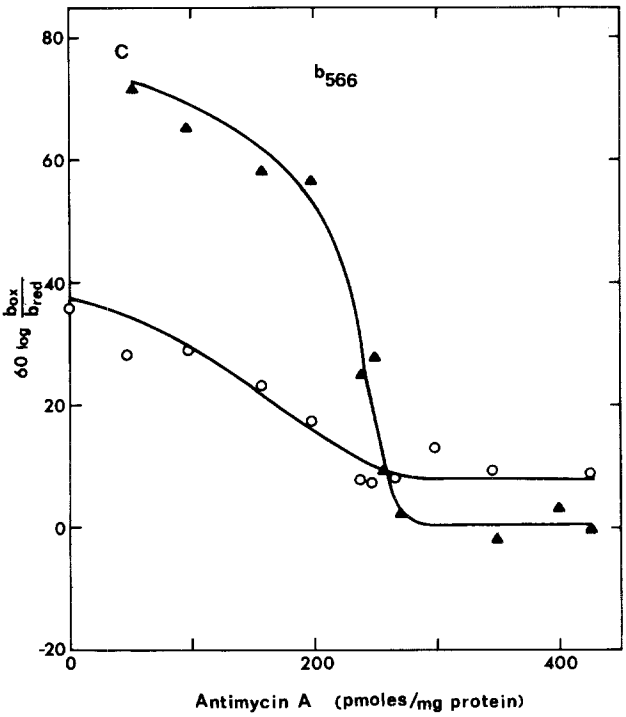


Fig. 2. Continued.

9), but this is not the case in the absence of antimycin. As will be shown, addition of ATP in the absence of antimycin initiates a reverse electron flux. Thus the response to the driving forces is also affected by the kinetic considerations, and quantitative interpretation should be avoided.

The Effect of the Applied Redox Potential on the Response of the b *Type Cytochromes of ATP*

In presence of saturating concentrations of ATP and antimycin, the extent of oxidation of cytochrome b_{561} is a function of the fumarate-to-succinate ratio. A Nernst plot of the redox state of cytochrome b_{561} in the absence and in the presence of ATP is given in Fig. 3. Cytochrome b_{561} behaves as a single electron carrier with a mid-potential of +70 mV, and in the presence of ATP its mid-potential assumes a value of +25 mV. The above calculation is based on the assumption that cytochrome b_{561} is a homogeneous population. An alternative interpretation is to assume that only a fraction of

total cytochrome b_{561} is oxidizable upon addition of ATP. On analyzing our experimental results referring only to the oxidized fraction, a nonlinear Nernst plot is obtained. Thus for the rest of this study, cytochrome *b* will be treated as a homogeneous population.

The response of cytochrome b_{566} to the addition of ATP was marginal. In some experiments no change in the redox state of b_{566} was measured, while in others a small oxidation (~9%) was noted. The reduction of cytochrome b_{566} in the presence of antimycin is enhanced by the presence of oxygen [18]. Even in the absence of oxygen the relaxation of reduced cytochrome b_{566} is not rapid unless PMS is added [19]. Because of that we repeated the titration under conditions where ascorbate plus TMPD were added in the absence of KCN. Thus anaerobiosis was achieved due to the activity of cytochrome oxidase. In order to allow a rapid relaxation of reduced cytochrome b_{566}, PMS (30 μM) was also present. The mixture was equilibrated in the cell compartment and anaerobiosis was identified as total reduction of cytochrome *c* or cytochrome oxidase. Succinate plus fumarate were added and once anaerobiosis was reestablished the absorbance spectrum was recorded. The same procedure was reported after addition of ATP. Under such controlled conditions the oxidation of cytochrome b_{561} was identical to that measured under standard condition while the redox state of b_{566} was constant and equal to that of the fumarate succinate couple, and in some cases a minor (~5%) reduction of cytochrome b_{566} was noted.

The Effect of pH on the ATP-Dependent Oxidation

Experiments as described in Fig. 3 were repeated at the pH values indicated in Fig. 4. The apparent mid-potentials of cytochrome b_{561} in the absence and in the presence of ATP are a linear function of pH in the range 6.0–8.5. The slopes of the lines are practically parallel and correspond to a redox reaction with stoichiometry of one proton per electron.

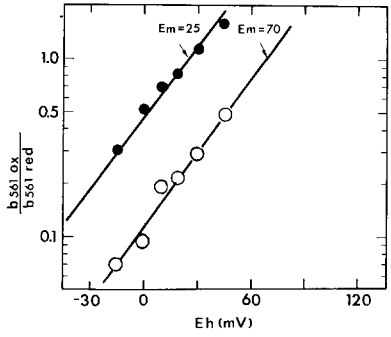


Fig. 3. The effect of ATP on the redox titration of cytochrome b_{561} in antimycin-inhibited submitochondrial particles. Experiments as in Fig. 1 were repeated with various fumarate: succinate ratios to give the redox potential indicated in the abcissa. The redox state of cytochrome b_{561} was calculated before (○) and after (●) addition of ATP as described in Materials and Methods.

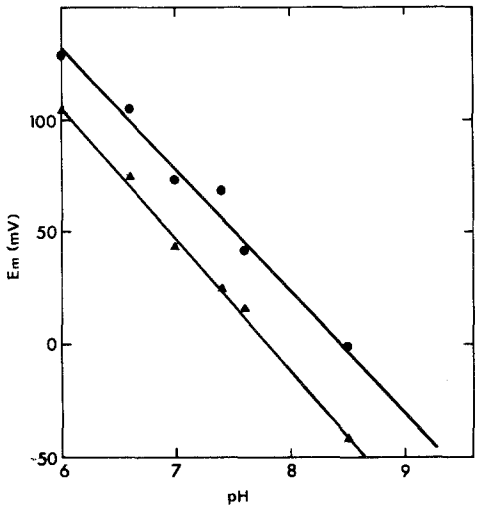


Fig. 4. The pH dependence of cytochrome b_{561} mid-potential as measured in the presence or absence of ATP for antimycin-inhibited submitochondrial particles. The experiment described in Fig. 3 was repeated at the indicated pH values. The E_m values measured in the absence (●) and in the presence (▲) of ATP are drawn with respect to pH. The redox potential of the fumarate: succinate couple was corrected for the pH as described under Methods.

The Driving Force for Oxidation of Cytochrome b_{561}

The extent of cytochrome b_{561} oxidation varies either with ATP concentration or phosphate potential (Fig. 5A and B). The response to ATP concentration follows a saturation curve. The ATP concentration which causes 50% of maximal oxidation is 25 μM.

Changing the phosphate potential of the reaction also modulates the magnitude of the oxidation. At a high phosphate potential ($\Delta G_{ATP} < -12$ kcal/mole) the oxidation reaches a maximal limiting value.

It is of interest to point out that measurable oxidation is obtained at such low phosphate potential (~6 kcal/mole) that even commercial ADP (usually contaminated by ~0.5% ATP) causes detectable oxidation. Prior treatment of the ADP solution with hexokinase plus glucose abolished the oxidation measured upon addition of ADP.

In order to investigate the nature of the driving force for the oxidation, we tried to distinguish between $\Delta\psi$ and ΔpH components of the proton motive force. The value of ΔpH was measured fluorimetrically [13] using 9-aminoacridine. Phosphorylating submitochondrial particle, suspended in 30 mM KCl, 50 mM Tris-chloride, and 5 mM $MgCl_2$ pH 7.4 (KCl medium),

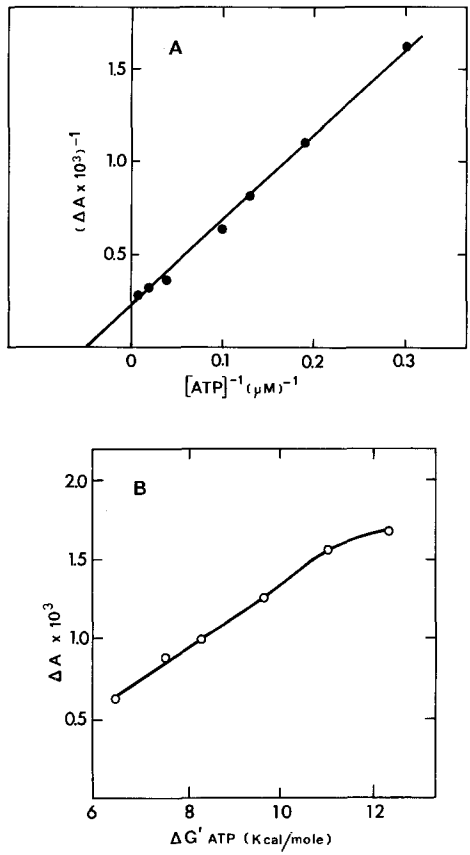


Fig. 5. The effect of ATP concentration (A) and phosphate potential (B) on the oxidation of cytochrome b_{561} in antimycin-inhibited submitochondrial particles. The experimental procedure in Fig. 1 was repeated, except that the concentration of ATP was varied or added together with ADP and phosphate to the indicated phosphate potential ($\Delta G^{\circ\prime}_{ATP} = -7.3$ kcal/mole). Cytochrome b_{561} oxidation was measured at 562.5–575 nm. Note the double reciprocal presentation in (A).

can easily reach 2.5–3 pH units at the expense of NADH oxidation or 2.4 units during ATP hydrolysis. Addition of Nigericin (1 μm) caused a complete recovery of the fluorescence. In the same buffer and in the presence of Nigericin, ATP caused the normal oxidation of cytochrome b_{561}. Similarly addition of Nigericin after addition of ATP did not reverse the oxidation of cytochrome b_{561}. Further support for ruling out ΔpH as the driving force for oxidation came from studies in sucrose-Tris-Mes buffer. This system, lacking a permeant anion, maintains a high $\Delta\psi$ value, but fails to demonstrate upon addition of ATP a fluorescence quenching of 9-aminoacridine. Still the normal oxidation of cytochrome b_{561} was measured in this buffer system even

in the presence of Nigericin. Moreover, as documented below, the addition of SCN^- to sucrose-Tris-Mes buffer completely reversed the oxidation of cytochrome b_{561} although it built up a pH gradient of 2 pH units (not shown).

Identification of $\Delta\psi$ as Driving Force for Oxidation

In order to quantitate the oxidation of cytochrome *b* with respect to $\Delta\psi$, we monitored the latter by measuring the spectral shift of safranine [11]. In submitochondrial vesicles the magnitude of the safranine signal is a linear function of $\Delta\psi$ in the range of 0–100 mV (positive inside) (Fig. 6).

In parallel, we determined the magnitude of $\Delta\psi$ built up during ATP hydrolysis. The same value was measured both by safranine (100 mV) and by flow dialysis (95 ± 10 mV). Thus as long as we keep the magnitude of the signal within the linear section of Fig. 6 and adhere to the standard concentration of the reactants, protein, and safranine concentrations, the magnitude of $\Delta\psi$ can be directly measured.

The correlation between the safranine signal and oxidation of b_{561} is given in Fig. 7. In this experiment the oxidation was induced by ATP in sucrose-Tris-Mes buffer and the extent of oxidation of cytochrome b_{561} and the spectral shift of the safranine was measured. This procedure was repeated in the presence of varying concentrations of permeant anions, SCN^- and NO_3^-. As seen in the figure, both parameters decay in parallel.

The correlation between $\Delta\psi$ and E_h is better emphasized in Fig. 8 where the two potentials are drawn with respect to each other. It is evident that whatever method was utilized to deenergize the membranes, FCCP, permeant anions, or blocking of ATPase, there is a linear correlation between the shift in mid-potential of cytochrome b_{561} and the measured $\Delta\psi$.

The linear correlation of the two parameters was also observed in kinetic

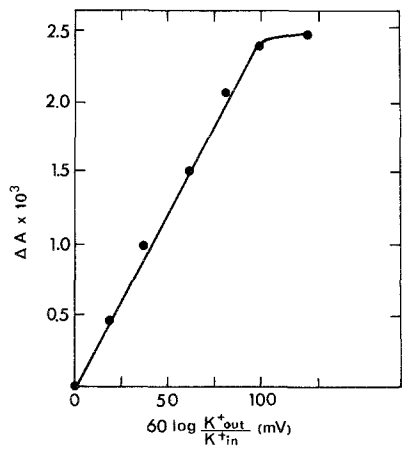


Fig. 6. Calibration curve of the safranine spectral change measured in submitochondrial particle by K^+ diffusion potential. ETP_H (1.5 mg/ml) were suspended in sucrose-Tris buffer containing 16 μM safranine reduced by 2 mM NaCN, 10 mM sodium fumarate, 1 mM sodium succinate, 4 mM sodium ascorbate, and 66 μM TMPD. The system was equilibrated for 5 min with 1 mM KCl and 1 μM valinomycin. Then the external K^+ concentration was changed by addition of various concentrations of KCl, and the increase in the absorbance at 511–533 nm was measured. The diffusion potential was calculated according to the Nernst equation

$$\Delta\psi = 60 \log \frac{[K^+_{out}]}{[K^+_{in}]}.$$

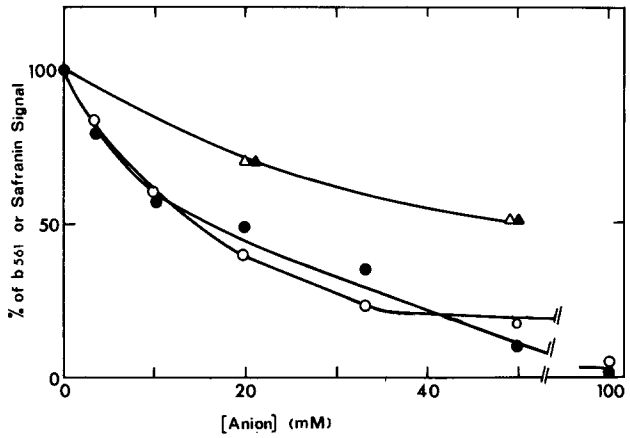


Fig. 7. The effect of permeant anions on the oxidation of cytochrome b_{561} and the magnitude of $\Delta\psi$ driven by ATP in antimycin-inhibited submitochondrial particles. ETP_H were inhibited by antimycin and reduced by the fumarate : succinate couple (10:1) in the presence of ascorbate, TMPD, and KCN as in Fig. 1. The effect of ATP on the oxidation of cytochrome b_{561} (solid symbols ●, ▲) or the spectral shift of safranine (open symbols ○, △) was measured in the presence of the indicated concentrations of KSCN (●, ○) or KNO_3 (▲, △). The oxidation of cytochrome b_{561} was measured at 562.5–575, and the spectral shift of safranine at 511–533 nm. The results are normalized with respect to the signal measured in the absence of permeant anions.

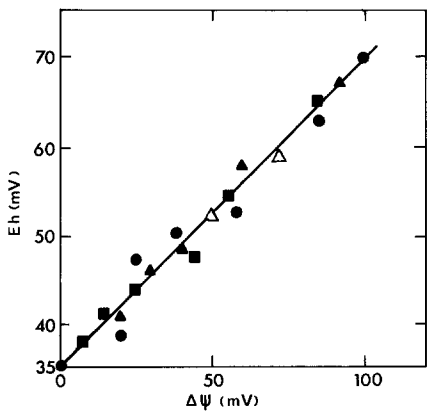


Fig. 8. Correlation between the redox potential of cytochrome b_{561} in antimycin-inhibited submitochondrial particles and the ATPase-driven $\Delta\psi$. The value of $\Delta\psi$ was measured by following the ATP-induced safranine spectral shift, using Fig. 6 as calibration curve. The highest measured value was corroborated by equilibrium dialysis. The redox potential (E_h) of cytochrome b_{561} was calculated from the redox state and the mid-potential as detailed in Methods. The results represent two kinds of experiments: △ and ● represent the level of the two potentials when measured at steady states in the presence of various concentrations of permeant anions [NO_3^- (△) and SCN^- (●)]. Results taken from Fig. 7. The rest of the points in this figure were taken from the experiment summarized in Table I. The magnitudes of the two potentials measured at the same time after addition of 1 μM FCCP (▲) or 1 μM oligomycin (■) are drawn with respect to each other.

Table I. The Correlation Between the Pseudo-First-Order Rate Constants for the Change in Redox State of Cytochrome b_{561} and the Safranine Spectral Shift During Buildup and Collapse of $\Delta\psi$ in Antimycin-Inhibited Submitochondrial Particles[a]

	Rate constant of response to ATP (sec^{-1})		Rate constant of response to collapse of $\Delta\psi$ (sec^{-1})		
Experiment number	Cytochrome b_{561}	Safranine spectral shift	$\Delta\psi$ collapsing agent	Cytochrome b_{561}	Safranine spectral shift
1	0.125	0.111	FCCP	0.055	0.068
2	0.111	0.14	oligomycin	0.083	0.125
3	0.125	0.14	hexokinase + glucose	0.078	0.067

[a]The oxidation of cytochrome b_{561} (measured at 561–575 nm) and the buildup of $\Delta\psi$ (measured by safranine) was measured kinetically as described in Figs. 2 and 6 respectively, showing pseudo-first-order kinetics. The collapse of the $\Delta\psi$ was achieved by FCCP (1 μM), oligomycin (1 μM), or by removal of ATP by hexokinase (100 units) plus glucose (1 mM). All kinetic responses were found to follow pseudo-first-order kinetics over 90% of the measured reaction.

studies (See Table I). In these experiments the kinetics of build up and relaxation of the oxidized state of cytochrome b_{561} was measured in parallel to the kinetics of appearance and collapse of $\Delta\psi$. $\Delta\psi$ was induced by addition of ATP and then abolished either by uncoupler, blocking of ATPase by oligomycin, or exhaustion of ATP by hexokinase plus glucose. As seen in Table I the kinetics of the decrease of E_h and $\Delta\psi$ are characterized by the same rate constants.

Finally we wish to point out that the calibration curve in Fig. 7 was obtained at a constant phosphate potential; thus though $\Delta\psi$ was varied, $\Delta\mu H^+$ was constant. As shown in Fig. 8 (Δ and $\bullet$) the oxidation of cytochrome b_{561} in this experiment varied linearly with $\Delta\psi$. On these grounds, we conclude that only the $\Delta\psi$ component of $\Delta\mu H^+$ is the driving force for the measured oxidation.

Discussion

The oxidation of cytochrome b_{561} reported in this study represents the interaction of the cytochromes with a single redox couple under the influence of an external coupled driving force generated by the hydrolysis of ATP. This oxidation should not be mixed with the effect of ATP on the redox state of the cytochrome *b* in mitochondria maintained in the presence of ascorbate as electron donor and fully oxidized pyridine nucleotides [15]. While the oxidation we observe can be classified as a static head [20] the system described by Wilson [15] approximates a level flow through two coupling sites, one at the *c*-*b*-Q level and the other through NADH dehydrogenase [21, 22].

Early reports on oxidation of cytochrome b_{561} in the presence of ATP and antimycin utilized submitochondrial particles [1, 3] in the presence of mixed substrates [1] or NADH at low potential donor, which interacts with cytochrome *c* through two coupling sites [3]. Later studies were carried out on intact mitochondria [2, 17] where the presence of endogenase substrate and the enzymes of the Krebs cycle impaired the ability to maintain a known redox potential in the system. Because of these reasons we prefer to employ submitochondrial particles where the oxidation of succinate is carried out by the bulk-facing *M* side of the membrane. Furthermore in the absence of fumarase no low-potential substrate (malate) can be formed. Thus we could carry out our experiments under well-defined redox potentials.

By poising the redox potential of the bulk over a wide range (-30 to $+30$ mV) we were able to monitor the effect of ATP when the initial redox state of the carriers under observation varied by ten times (see Fig. 3). This allowed quantitation of the oxidation in absolute units of the shift of redox potential expressed in millivolts.

The wavelength of maximal absorbance change corresponding to the ATP-induced oxidation is 562.5–563 nm. Thus we observe oxidation of both cytochrome b_{561} and b_{566}. In order to analyze the response of each cytochrome we repeated the procedure of Wilson [15] to determine the contribution of each cytochrome at 561 and 566 nm.

In analyzing our results we utilized the parameters we obtained. Yet it should be emphasized that repeating the calculation using Wilson's parameters only slightly affected the magnitude of the measured potential but had no effect on the change in the redox potential (ΔE_h).

In the presence of antimycin the redox state of cytochrome b_{566} is sharply dependent on the redox state of c_1 [18]. Thus in order to avoid perturbation of the redox state of cytochrome b_{566} by that of c_1, we maintained the carriers on the oxygen side of cytochrome c_1 in a fully reduced state by a combination of KCN, ascorbate, and TMPD.

In the absence of ATP, the system we employed can be regarded as being in true equilibrium. On the other hand, once ATP is added only a static head is established [8]. Still, as we limited our observation to the flux which came to a standstill (electron flux), the utilization of the equilibrium equation is justified.

The redox state of each *b* type cytochrome was measured in the presence and absence of antimycin and in the absence and presence of ATP. Of these four defined states, the redox state measured in the presence of ATP and in the absence of antimycin is not suitable for any quantitative analysis. This state represents reverse electron transport for cytochrome c_1 (or cytochrome *c*) to the quinone level which then equilibrates with the fumarate succinate couple. As the rate-limiting step in this sequential reaction is not defined, quantitative conclusions cannot be drawn. On the other hand, the redox states

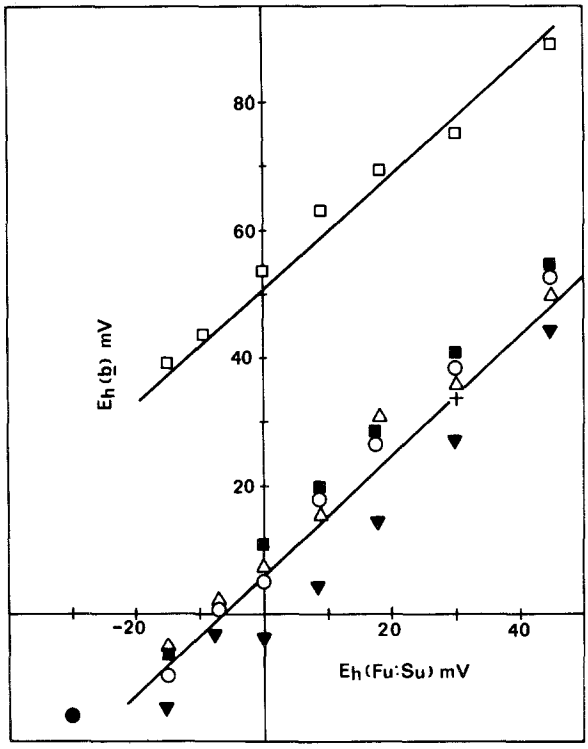


Fig. 9. The correlation of the measured potential of the *b* type cytochromes with the redox potential of fumarate : succinate in energized and nonenergized submitochondrial particles. ETP_H (1.5 mg/ml) were treated with 2 mM KCN, 4 mM ascorbate, and 66 μM TMPD. The redox state of cytochromes b_{561} and b_{566} were calculated as described in Methods and are drawn with respect to the redox potential of the fumarate:succinate couple. The experiments were carried out either in the absence or presence of antimycin in energized (1 mM ATP) or deenergized particles. ○, cytochrome b_{561} in the absence of both antimycin and ATP; △, cytochrome b_{561} in the presence of antimycin and in the absence of ATP; □, cytochrome b_{561} in the presence of ATP and antimycin; +, cytochrome b_{561} in the presence of ATP and absence of antimycin; ●, cytochrome b_{566} in the absence of antimycin or ATP; ▼, cytochrome b_{566} in the presence of antimycin and absence of ATP; ■, cytochrome b_{566} in the presence of ATP and antimycin.

measured in the presence of antimycin and ATP are states without electron flux and will be discussed below.

The state of equilibrium (or quasi-equilibrium) of the redox components is established when the electrochemical potential of the electron [8] is equal for all reactants. In our system there are three components and two phases: bulk aquose where the redox system is maintained constant by fumarate and succinate and is regarded as the reference in our case; the membranal phase which is permeable to electrons either through the cytochromes or the quinone and the internal aquase phase which assumes, with respect to the

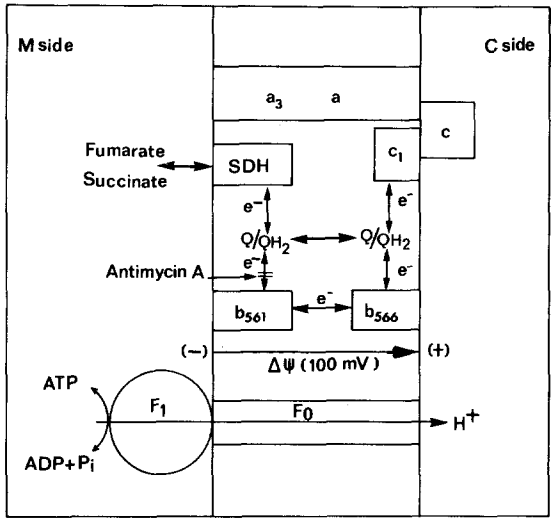
bulk, an electric potential of +100 mV (Scheme I). The correlation between the redox potential, calculated from the redox state of the cytochromes and the corresponding E_h value, and the fumarate : succinate couple is given in Fig. 9.

In the absence of antimycin and ATP, b_{561} and b_{566} are in redox equilibrium with the succinate : fumarate couple. In this case $\Delta\psi = 0$. The electrical potential does not affect the electrochemical potential of the electrons, and the redox state of the carriers on both sides of the membrane are in accord with the expected value.

Once antimycin is added, the mid-potentials of cytochromes b_{566} and b_{561} are shifted respectively from −62 (−38 at pH 7 [17]) to +30 mV and from 50 to 70 mV. This accounts for the fact that the equilibrium of cytochrome b_{566} with the donor couple becomes experimentally observable. As seen in Fig. 9 under these conditions the redox state and the corresponding E_h values measured for the *b* type cytochromes are in accord with the potential set by the fumarate : succinate couple.

Once ATP is added the situation is changed. Cytochrome b_{566}, located on the opposite site of the membrane with respect to the reference couple (Scheme I), still assumes the redox potential of the bulk. On the other hand cytochrome b_{561} which is closer to the bulk, reflects a major shift of the electron electrochemical potential which it senses. The magnitude of this discrepancy is ~40 mV and is not sensitive to pH.

The oxidation of cytochrome b_{561} measured under these conditions is an



Scheme I

energy-dependent reaction. The oxidation is sensitive to uncouplers (Fig. 1). It is not measured in the presence of oligomycin and its magnitude is proportional to the phosphate potential applied on the system (Fig. 5B).

The driving force for the oxidation of cytochrome b_{561} can be the proton motive force or only one of its components ΔpH or $\Delta\psi$ (Scheme I). The involvement of ΔpH in the oxidation of cytochrome b_{566} can be ruled out because of the following reasons. Oxidation of cytochrome b_{561} measured in the sucrose Tris-acetate buffer, where no ΔpH was detected by 9-aminoacridine, is compatible with that measured in KCl medium where $\Delta pH = 2.5$. Addition of Nigericin collapses the ΔpH built up in KCl medium without affecting the magnitude of the oxidation expressed either as a percent of cytochrome b_{561} or when calculated in millivolts. Moreover, addition of KCNS, which replaces $\Delta\psi$ by ΔpH, completely abolishes the ATP-dependent oxidation of cytochrome b_{561}.

The dependence of oxidation on $\Delta\psi$ is demonstrated by the following facts. There is a linear correlation between $\Delta\psi$ [measured by safranine and calibrated by K^+ gradient (Fig. 6) or flow dialysis] and the shift of E_h sensed by b_{561} (Fig. 8). Furthermore the kinetics of the buildup and decay of $\Delta\psi$ (measured by safranine) and ΔE_h for cytochrome b_{561} are characterized by the same time constants (Table I). Thus the identification of $\Delta\psi$ as the driving force for the oxidation is supported by equilibrium and kinetic measurements.

Once the oxidation of cytochrome b_{561} by the electric component of the proton motive force is established, the explanation of its oxidation becomes a straightforward consequence of the Q cycle, or any other mechanism which stipulates that the interaction of b_{561} with Q is antimycin sensitive and locates b_{561} and b_{566} on the *M* and *C* sides respectively.

In the absence of antimycin, no relative change in the redox state of cytochrome b_{561} or b_{566} is expected as both are in equilibrium with the quinone, an uncharged mobile carrier [23]. ATP-dependent $\Delta\psi$ will change the electrochemical potential of the electron [8], converting the *C* side cytochrome b_{566} to an oxidant with respect to cytochrome b_{561}. Still this gradient in electrochemical potential will be dissipated into a flux by the following mechanism. Cytochrome b_{566} is located in the membrane *C* side together with some of the quinone, and their redox equilibrium is not affected by $\Delta\psi$. Consequently any enhanced reduction (driven by $\Delta\psi$) of cytochrome b_{566} is matched by a comparable reduction of some of the quinone. As ubiquinone is an uncharged mobile carrier, any gradient in its redox state (among different loci in the membrane) will dissipate by diffusion till quasi-equilibrium is established.

The same consideration applies for cytochrome b_{561}. Its redox equilibrium with some of the membrane quinone is not affected by $\Delta\psi$, and as the

redox state of ubiquinone in different loci is equalized (or nearly so) by semiequilibrium, cytochromes b_{561} and b_{566} will assume close redox potentials (see Fig. 9), reflecting the various rate constants associated with the quasi-equilibrium state.

As a matter of fact, such open systems (equilibrated with two redox couples, fumarate : succinate and ascorbate TMPD) function as a coupling system converting the proton motive force into an uphill reverse electron transport.

In contrast to the above case, once antimycin is present, the oxidation of cytochrome b_{561} by QH_2 (M side) is inhibited and the magnitude of the oxidation in millivolts should be fully equated with the electrical gradient between b_{561} and its C side oxidant (most probably b_{566}). This accounts for the oxidation of b_{561}. Under these conditions cytochrome b_{566} senses an electrochemical potential of E_h 566 = E_h out + 100 mV and should react in a highly reduced state. But as its eqilibrium with Q (C side) is not affected by $\Delta\psi$, it will equilibrate in a redox reaction with the quinone which, because of its mobile nature, maintains the redox protential of the M side. Thus we account for the oxidation of b_{561} without corresponding the shift in the redox state (or redox potential) of cytochrome b_{566}.

It is of interest to evaluate the effect of the acidification of the inner space (C side) of the vesicles on the redox state of the b type cytochromes. Generation of ΔpH will alter the mid-potential of these components facing the C side; thus at constant electrochemical potential of electrons (as poised by the succinate : fumarate couple) their redox state will vary. Cytochrome b_{561} faces the M side of the membrane (see scheme I) and thus acidification of the inner space will not affect its redox state, in accord with our observations.

The redox potential of cytochrome b_{566} is pH dependent only above pH 6.9, and its E_m varies with a slope of 60 mV/pH unit [7]. Thus under our experimental conditions (where the initial pH = 7.4), the maximal shift in the mid-potential of cytochrome b_{566} cannot exceed ~30 mV (corresponding to 0.5 pH unit), whatever is the magnitude of ΔpH. A 30-mV shift of mid-potential is large enough to be observed as enhanced reduction of cytochrome b_{566}. Still, under no condition (in presence of antimycin) did we observe an ATP-dependent reduction of cytochrome b_{566}. This fact might suggest that in submitochondrial particles cytochrome b_{566} exchanges protons with the M side of the membrane rather than with the C side.

The last point for consideration is the ratio between $\Delta\psi$ and the resulting oxidation by cytochrome b_{561}. As seen in Fig. 8, the redox potential applied on b_{561} (in presence of antimycin) increases above the potential of the bulk (fumarate : succinate) in a linear function of $\Delta\psi$. The slope of this linear function (0.35 ± 0.05) is constant over the whole range of measured values of $\Delta\psi$. As $\Delta\psi$ was measured by a double calibration system (K^+ gradient, Fig. 6,

and equilibrium dialysis), the less-than-unity value of the slope indicates that only a fraction of the total electric potential gradient affects the redox equilibrium of cytochromes b_{566} and b_{561} [8]. Knowledge about the electric potential profile across the membrane is presently limited. Still, based on our measurements, it seems that the relative location of b_{566} and b_{561} in the membrane is such that only a fraction of the total electric field affects their relative redox state.

References

1. P. C. Hinkle, R. A. Butow, E. Racker, and B. Chance. *J. Biol. Chem.*, **242,** (1967) 5169–5173.
2. T. Flatmark and J. I. Pedersen, *Biochim. Biophys. Acta,* **325** (1973) 16–28.
3. E. C. Slater and I. Y. Lee, in Oxidases and Related Redox Systems, T. E. King, R. K. Morton, and M. Morrison, eds., Vol. 2, University Park Press, Baltimore (1973), pp. 823–850.
4. M. K. F. Wikstrom, *Biochim. Biophys. Acta,* **301** (1973) 155–193.
5. P. Mitchell, *FEBS Lett.* **56** (1975) 1–6.
6. B. L. Trumpower, *Biochem. Biophys. Res. Commun.*, **70** (1976) 73–80.
7. D. F. Wilson, M. Erecinska, J. S. Leigh, and M. Koppelman, *Arch. Biochem. Biophys.*, **151** (1972) 112–121.
8. D. Walz, *Biochim. Biophys. Acta,* **505** (1979) 279-353.
9. R. L. Ringler, S. Minakami, and T. P. Singer, *J. Biol. Chem.*, **238,** (1963) 801–810.
10. M. Hansen and A. L. Smith, *Biochim. Biophys. Acta,* **81** (1964) 214–222.
11. K. E. O. Akerman and M. K. F. Wikstrom, *FEBS Lett.* **68** (1976) 191–197.
12. S. P. Colowick and F. C. Womack, *J. Biol. Chem.*, **244,** (1969) 774–777.
13. S. Papa, B. T. Storey, M. Lorusso, C. P. Lee, and B. Chance, *Biochim. Biophys. Acta,* **292** (1973) 20–38.
14. H. Rottenberg and C. P. Lee, *Biochemistry,* **14** (1975) 2675–2680.
15. D. F. Wilson and M. Erecinska, *Arch. Biochem. Biophys,* **167** (1975) 116–127.
16. H. Borsook and H. Scott, *J. Biol. Chem.*, **92** (1931) 535–557.
17. M. Erecinska and D. F. Wilson, *Arch. Biochem. Biophys.*, **174** (1976) 143–157.
18. H. Baum and J. S. Rieske, *Biochem. Biophys. Res. Commun.* **24** (1966) 1–9.
19. M. K. F. Wikstrom and J. A. Berden, *Biochim. Biophys. Acta.*, **283** (1972) 403–420.
20. O. Kedem and S. R. Coplan, *Trans. Faradrey. Soc.*, **61** (1965) 1897–1911.
21. M. Gutman, M. Beinert and T. P. Singer, in *Electron Transfer Chains and Oxidative Phosphorylation,* E. Quagliariello, S. Papa, F. Palmieri, E. C. Slater, and N. Siliprandi, eds., North-Holland, Amsterdam (1976) pp 55–62.
22. H. Rottenberg and M. Gutman, *Biochemistry,* **16** (1977) 3220–3227.
23. G. Hauska in *Membrane Bioenergetics,* L. Packer, G. C. Papageorgiou, and A. Trebst, eds., Elsevier/North-Holland (1977) pp. 177–187.