Bioenergetics (1970) **1**, 45–60.

Effect of Membrane Potential on Equilibrium Poise Between Cytochrome *a* and Cytochrome *c* in Rat Liver Mitochondria

Peter Hinkle* and Peter Mitchell

Glynn Research Laboratories,
Bodmin, Cornwall, England

Abstract

The object of this work was to test the suggestion that the equilibrium poise between cytochrome *a* and cytochrome *c* in mitochondria might be influenced by the membrane potential.

1. The midpoint potentials of cytochromes $(c + c_1)$ and cytochrome *a* (CO present) were found to be 250 mV and 245 mV, respectively, by equilibrating rat liver mitochondria with mixtures of ferrocyanide and ferricyanide anaerobically in presence of antimycin A and measuring the redox state of the cytochromes spectrophotometrically. In absence of CO, cytochrome oxidase gave an anomalous redox titration curve with a "midpoint" at about 275 mV.

2. When the mitochondria were equilibrated with ferricyanide/ferrocyanide, the redox poise of cytochrome *a* (CO present) and of cytochromes $(a + a_3)$ but not of cytochromes $(c + c_1)$ was dependent on the sign and magnitude of the membrane potential developed by treating the mitochondria as follows: by adding ATP, by changing the composition of the suspension medium so as to vary the Donnan or Nernst potential, by adding valinomycin in a medium of low K^+ ion content, or by adding a pulse of acid or alkali when the membrane was made permeable to protons with FCCP.†

3. The findings agree with the suggestion that the respiratory chain is arranged across the cristae membrane with cytochromes c_1 and *c* in contact with the outer phase and cytochromes *a* and a_3 plugged through, so that the equilibrium distribution of electrons between the *c* and *a* cytochromes is influenced by the electric field across the membrane.

Introduction

The spectrophotometric studies by Chance and Williams[1] and by Klingenberg and Schollmeyer[2] showed that the addition of ADP to mitochondria respiring in State 4 (without phosphate acceptor but with substrate and phosphate) caused the redox poise of cytochromes $(a + a_3)$ to become more reduced while that of cytochromes $(c + c_1)$ became more oxidized. This "crossover" effect was taken to mean that there is a "coupling site" in the cytochrome *c*–cytochrome oxidase region of the respiratory chain. The reversal by ATP of electron transfer between cytochrome *c* and cytochrome *a*, observed by Caswell,[3] strongly supports the view that electron transfer between cytochrome *c* and cytochrome oxidase is coupled to phosphorylation. Muraoka and Slater[4] have recently criticized the

* Present address: Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850, U.S.A.

† Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone; EGTA, ethylene glycol-bis (aminoethyl)-tetraacetic acid; cyt, cytochrome; val, valinomycin.

interpretation of "crossover" data; and doubts have also been expressed[5] about the feasibility of coupling phosphorylation to electron transfer between cytochrome *c* and cytochrome *a* because the midpoint potential difference is only some 20 or 30 mV, compared with the 250 mV[6,7] or possibly more[8,9] required to reverse the ATPase reaction. Nevertheless, there is general agreement that the essential stoichiometric and thermodynamic requirements could not be met unless electron transfer between cytochrome *c* and oxygen were coupled to the reversal of the ATPase so as to give at least one ATP molecule per electron pair equivalent traversing this part of the respiratory chain. The work of Minnaert[10] and of Horio and Ohkawa[11] suggests that the midpoint potential of cytochrome a_3 might be much more positive than that of cytochrome *a*. The cytochrome a_3 (and possibly other components of the oxidase complex) might thus provide the required energetic potential span relative to cytochrome *c*.

We wish to focus attention on the possibility that, as suggested by Mitchell,[12] the coupling of phosphorylation to electron transfer in the cytochrome oxidase region depends on the electric current-carrying arrangement of the cytochrome c_1 to cytochrome a_3 part of the respiratory chain across the cristae membrane. According to this view, the membrane potential (positive outside) across the osmotic barrier or M phase of the cristae membrane should have a pulling effect on the electrons from cytochrome a_3 (near the inner surface of the M phase) to cytochrome c_1 (near the outer surface); and this effect should result in shifts of the poises of cytochromes *a* and a_3 in equilibrium with cytochromes *c* and c_1, which should be quantitatively related to the local electric potential differences between the haem groups of the cytochromes, as previously discussed.[13,14] Experimental studies of such shifts of poise are interesting both in the context of the coupling mechanism and in the context of the biochemical characterization of the cytochrome oxidase region of the respiratory chain.

The experiments described in this paper were undertaken to obtain information about the relative poises of cytochrome *a* and cytochromes $(c + c_1)$ in rat liver mitochondria under conditions such that these cytochromes are near equilibrium with a ferricyanide/ferrocyanide redox buffer in the suspension medium; and to test the expectation that changes of membrane potential (but not of pH differential) should cause shifts in the poises of cytochrome *a*, cytochrome *c*, and cytochrome c_1, depending on the average depths of their haem groups below the outer surface of the M phase.

Materials and Methods

The methods of isolating the rat liver mitochondria, measuring pH and pK with glass electrodes, and preparing and dispensing anaerobic reagents were as previously described.[15–17] In experiments with the carbon monoxide complex of cytochrome oxidase, the degassed suspension medium was saturated with CO by bubbling with the pure gas from a cylinder (Air Products Ltd., Hythe, Southampton, Hampshire) for 10 min. Anaerobic or CO-saturated media were dispensed into the reaction vessel from 50 ml glass syringes fitted with flexible polythene "needles" made by pulling out thin-walled 3 mm bore polythene tubing in a low-temperature flame.

The extent of reduction of cytochromes $(c + c_1)$ and cytochrome *a* or $(a + a_3)$ were measured with a Phoenix double beam spectrophotometer (Phoenix Precision Instrument Co., Philadelphia, Pennsylvania, U.S.A.) using the absorbance differences $A_{550-540}$ and

$A_{605-630}$, respectively. The closed reaction vessel, containing the mitochondrial suspension at a density corresponding to 5–6 mg of protein/ml, had a light path of 1 cm; and in the fully reduced condition, absorbance differences in the region of 0·02 and 0·01 were recorded for cytochromes *c* and *a*, respectively.

The amplified signals from the H^+ ion- and K^+ ion-sensitive electrodes and from the double beam spectrophotometer were fed into a multichannel strip chart recorder (Oscillograph type 5-127 of Bell and Howell Ltd., Basingstoke, Hants, England).

Reaction Vessel

In order to permit continuous spectrophotometric, pH, and pK measurement during titrations of anaerobic or carbon monoxide-saturated mitochondrial suspensions, a vessel similar to the anaerobic glass titration vessel previously described[15] was constructed from a quartz cuvette 3 cm wide, 2 cm high and of 1 cm light path. The contents of the cuvette were continuously stirred by a glass propeller formed on the end of the plunger of a small all-glass hypodermic syringe. Part of the barrel of the syringe was fitted into a ground glass socket in the end of the cuvette and acted as a practically water-tight bearing for the plunger which was inserted through it. The plunger (carrying the propeller) was rotated at about 500 rpm by a small electric motor connected by a flexible plastic sleeve. The interior of the cuvette was accessible only through a glass capillary tube cemented vertically to the top and surmounted by a small glass reservoir. The cuvette was filled and emptied, and reagents were dispensed into it via fine plastic or glass "needles" designed to be inserted through the capillary. The capacity of the cuvette was 5·0 ml, and the temperature of the cell compartment was regulated at 25° by water circulated from a constant-temperature water bath.

Reagents

In addition to the standard acid and alkali and other reagents prepared as previously described,[15–17] standard ferricyanide and ferrocyanide solutions were freshly made up each day from Analar grade potassium and sodium salts and dispensed, like the standard acid and alkali, at suitable concentrations from calibrated micrometer syringes. Cytochrome *c* was obtained from Sigma London Chemical Co. Ltd. (London, S.W.6).

Estimation of Membrane Potential

Membrane potentials ($\Delta\psi$) were calulated as before[17] by estimating the pH or pK difference (ΔpH or ΔpK) across the cristae membrane in presence of FCCP (1 μM) or valinomycin (100 μg/g of mitochondrial protein), respectively, using the Nernst equation, which can be written in the simple form

$$\Delta\psi = -59\Delta\text{pH} \tag{1}$$

or

$$\Delta\psi = -59\Delta\text{pK} \tag{2}$$

where $\Delta\psi$ refers to the membrane potential (written positive when the outer phase is relatively positive), the differences ΔpH or ΔpK refer to outer phase minus inner phase values, and under conditions where H^+ ions or K^+ ions, respectively, are equilibrated across the membrane. The highly specific actions of FCCP and valinomycin in inducing

permeability to H^+ ions and K^+ ions, respectively, have been discussed previously[12–14] and have recently been further verified in the case of the cristae membrane of rat liver mitochondria.[17]

Rationale of Measurement of Redox Potentials of Cytochromes

We have used the orthodox method of estimating total cytochrome by the maximum absorbance difference change at the appropriate wavelength pair on going from a very oxidized to a very reduced state.[1] The percentage reduction of cytochrome is correspondingly estimated as percentage maximal absorbance difference. In the case of cytochrome c and cytochrome c_1, the absorbance difference $A_{550-540}$ was taken to define the total quantity of ferrous haem of cytochromes $(c + c_1)$, and the percentage reduction correspondingly refers to the percentage of the total haem reduced in cytochromes $(c + c_1)$ in this work. The fact that the redox titration curve based on $A_{550-540}$ corresponded fairly closely to the theoretical curve for a one-electron transfer (Fig. 1) indicated that this procedure was a valid approximation.

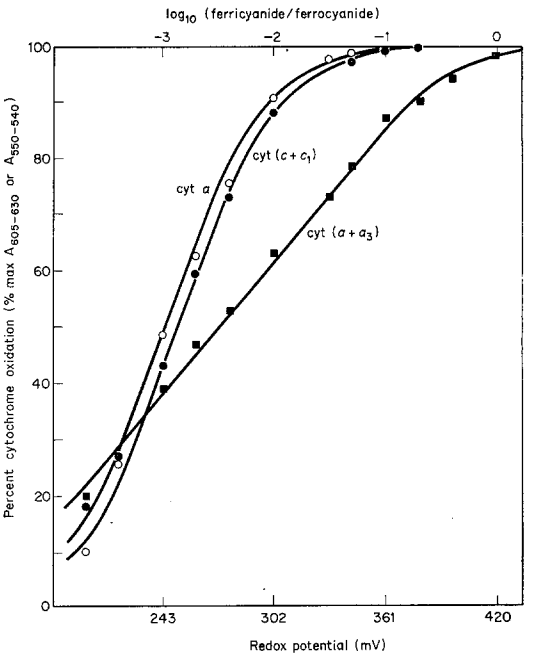

Figure 1. Redox titrations of cytochromes $(c + c_1)$, cytochrome a, and cytochromes $(a + a_3)$ in whole mitochondria. The mitochondria (5·0 mg of protein/ml) were suspended in anaerobic 135 mM KCl–25 mM sucrose medium containing 3 mM glycylglycine at pH 7·0, 1 μM FCCP, 0·2 μg of valinomycin/ml, 0·3 μg of antimycin A/ml, and 5 mM $K_4Fe(CN)_6$. The poises of the cytochromes were measured spectrophotometrically during stepwise titration with $K_3Fe(CN)_6$ as described in Materials and Methods. The medium was saturated with CO for the titration of cytochrome a. The curves for cytochromes a and $(c + c_1)$ represent theoretical single electron transfer titrations.

In the case of cytochrome a in normal cytochrome oxidase, the absorbance difference $A_{605-630}$ may or may not define the quantity of a haem group that is in the ferrous form. As shown for the isolated cytochrome oxidase,[10] and verified here for the oxidase in intact mitochondria (Fig. 1), spectrophotometric redox titrations of "cytochrome a" based on $A_{605-630}$, do not correspond to the one-electron transfer expected of a ferric to ferrous haem transition, but give a slope near the midpoint that would correspond to about a half-electron transfer. This may be accounted for either by a dependence of effective midpoint potential of cytochrome a on the redox state of other components of the system, or by a contribution of components other than cytochrome a to the value of $A_{605-630}$. In particular, it has been suggested that cytochrome a_3 contributes significantly to the absorbance difference $A_{605-630}$.[18] On the other hand, in the carbon monoxide complex of cytochrome oxidase, the CO is combined with the haem of cytochrome a_3 in the reduced state,[19] and the cytochrome a of both the isolated oxidase[20] and the oxidase in intact mitochondria (Fig. 1) gives a normal one-electron transfer redox titration based on the value of $A_{605-630}$. Thus, there is little doubt that the absorbance difference $A_{605-630}$

represents the oxidoreduction state of the haem of cytochrome *a* in presence of carbon monoxide. Most of the measurements described in this paper have been done in presence of carbon monoxide so that the $A_{605-630}$ values can be interpreted in terms of the redox state of the haem of cytochrome *a*. In the presence of CO, the midpoint potential of cytochrome *a* was found to be 245 mV, whereas the centre of the anomalous titration curve based on $A_{605-630}$ in absence of CO was considerably more positive. Since the cause of the anomalous shape of the titration curve in absence of CO is not known, the meaning of the apparent shift of "midpoint" potential is not clear. To cover the possibility that the changes of the poise of cytochrome *a* at a fixed poise of cytochromes $(c + c_1)$ might have occurred only when the system had been changed by the presence of CO, we did some experiments on the changes of $A_{605-630}$ in absence as well as in presence of CO, and have been able to verify that qualitatively similar changes in the absorbance difference $A_{605-630}$ occur in either case. All statements concerning the redox poise of cytochrome *a* in this paper refer to the system with CO present.

The experimental procedure adopted in this paper depends on the fact, discussed previously,[13, 14] that the oxidoreduction potential as usually measured in condensed systems is equivalent to the chemical potential of the electron at zero electric potential.[21] When two regions of a system are at the same total electron potential, having reached equilibrium because of the presence of electron carriers, a difference of electric potential (meaning electrostatic potential) between the two regions is compensated by an equal and opposite difference of oxidoreduction potential. According to the sign convention generally in use,[21] a region of negative electric potential has a positive or oxidizing oxidoreduction potential relative to a region at zero potential with which it is in electronic equilibrium. Thus,

$$E'' = E' - \delta\psi \tag{3}$$

where E' and E'' are the oxidoreduction potentials at electric potentials of zero and $+\delta\psi$, respectively. Similarly,

$$E''_m = E'_m + \delta\psi \tag{4}$$

where E'_m is the midpoint potential of an electron-carrying couple as normally defined,[21] and E''_m is the apparent midpoint potential of the same couple when in a region at an electric potential $\delta\psi$ higher than the reference system with which it is in electronic equilibrium.

The utilization of this rationale depends on obtaining equilibrium, or near equilibrium, conditions in the part of the respiratory chain system under observation. The work of Pressman,[23] Jacobs and Sanadi,[24] and Estabrook[25] showed that ferrocyanide and ferricyanide react well with the terminal part of the respiratory chain at some point or points on the oxygen side of the antimycin-sensitive site, probably at cytochrome *c*. In our main experimental procedure, the terminal part of the respiratory chain of rat liver mitochondria was isolated with antimycin A, and oxygen was excluded from the mitochondrial suspension. The terminal cytochromes were poised in the intact mitochondria using ferricyanide/ferrocyanide as a non-permeating[22] oxido-reduction buffer; and the effect of changing the membrane potential ($\Delta\psi$) on the poise of cytochromes $(c + c_1)$ and cytochrome *a* relative to that of the redox buffer in the suspension medium (at zero electric potential) was measured spectrophotometrically. In order to estimate the actual midpoint potential E'_m, as distinct from the apparent midpoint potential E''_m, we have taken

advantage of the fact that the electric potential difference across the cristae membrane is near zero in a 150 mM KCl medium or in 135 mM KCl medium containing 5 mM sodium or potassium ferrocyanide at pH 7 (ref. 17 and supplementary unpublished observations by P. Mitchell and J. Moyle).

Redox Titrations of Cytochromes

The anaerobic mitochondrial suspension was equilibrated with 5 mM ferrocyanide until the $A_{550-540}$ and $A_{605-630}$ values indicated complete reduction of the terminal cytochromes (about 1 min). Antimycin A was added anaerobically to minimize effects of endogenous reductants. The suspension was then titrated stepwise with anaerobic solutions of ferricyanide (5 mM or 250 mM) to cover the required range of redox potentials, and the corresponding stepwise changes of the absorbance differences $A_{550-540}$ and $A_{605-630}$ were followed with the spectrophotometer. A slow drift of the absorbance differences towards reduction, which was more pronounced at lower ferricyanide concentration and at more alkaline pH, was attributable to incompleteness of inhibition of electron transfer from endogenous substrates to ferricyanide in presence of antimycin A;[26] but at the usual rate of the stepwise titration the extent of the drift corresponded to an insignificant change of the ferricyanide/ferrocyanide poise. The completeness of redox equilibration of the cytochromes, even when additions of ferricyanide were made at 5 sec intervals, was indicated by the fact that if the titration were interrupted at any point by the injection of 10 μl of air-saturated saline, the value of $A_{550-540}$ or $A_{605-630}$ showed transient oxidation followed by reduction which was complete within 3 sec. We used suspension media containing at least 50 mM KCl or choline chloride for the redox titrations because equilibration with the ferricyanide/ferrocyanide redox buffer was more sluggish at low salt concentration.

The titration of the *c* cytochromes (and indirectly of cytochrome *a*) with the ferricyanide/ferrocyanide system suffers from the disadvantage that the midpoint potentials of these cytochromes are some 170 mV more negative than the midpoint potential of the poising system. Thus, as shown by Fig. 1, the ferricyanide/ferrocyanide ratio at the midpoint of the cytochrome *a* and cytochrome $(c + c_1)$ titrations is only slightly greater than 10^{-3}; and the redox buffering power of the ferricyanide/ferrocyanide system is low in this region, compared with what it is at higher potentials. This disadvantage was partly overcome by using a high concentration of ferrocyanide (generally 5 mM), so that the concentration of ferricyanide at the midpoint of the titrations of cytochrome *a* and cytochrome $(c + c_1)$ was generally in the region of 5 μM, which was about five times the concentration of each cytochrome in the mitochondria. It is obvious that the absolute redox potentials corresponding to the parts of the titration curves on the reduced side of the midpoint are subject to considerable error compared with those on the oxidized side. The midpoint redox potentials given for cytochrome *a* and cytochrome $(c + c_1)$ in this paper were obtained from the midpoints of theoretical redox curves (for single electron transfer) fitted on the experimental points, as shown in Fig. 1, and in this, and in other similar titrations, it was evident that the points on the reducing part of the curve (i.e. at redox potentials below +240 mV) showed more scatter than those on the oxidizing part, and tended to be displaced from the theoretical curve towards more negative redox potentials. Therefore, the oxidizing regions of the curves have been allowed to play a dominant part in fixing the values of the midpoint potentials.

It is well established that isolated cytochrome *c* and cytochrome *c* in the respiratory chain readily equilibrate with the ferricyanide/ferrocyanide system;[10, 21, 23-25] and in the present work, the reversibility of the poising effect of the ferricyanide/ferrocyanide system on cytochromes $(c + c_1)$ and cytochrome *a* was demonstrable, as mentioned above, in the region of the midpoint potentials of the cytochromes as well as elsewhere in the titrations.

In case any doubt should remain concerning the effectiveness of the ferricyanide/ferrocyanide system for poising cytochrome *c* under the conditions of our experiments, it is important to bear in mind that our main object was to study changes in the equilibrium poise between cytochrome *a* and cytochrome *c* under various conditions. The ferricyanide/ferrocyanide redox buffer was employed with the intention of fixing the redox potential of cytochrome *c* and enabling us to observe shifts in the poise of cytochrome *a* at a fixed poise of cytochrome *c* when conditions were varied so as to vary the membrane potential. Our experimental verification that the cytochrome $(c + c_1)$ poise did not change under the various conditions imposed to vary the membrane potential in our experiments (see Results and Discussion) confirmed that the intention of maintaining this poise constant was actually achieved.

The redox potentials of the known ratios of ferricyanide to ferrocyanide present in a given suspension medium during the titrations were determined from the normal theoretical curve relating potential to concentration ratio, using a spectrophotometric redox titration of 2 μM cytochrome *c* with ferricyanide/ferrocyanide in the same medium to obtain the effective midpoint potential of the ferricyanide/ferrocyanide couple, taking a value of 255 mV for the midpoint potential of cytochrome *c*, irrespective of the medium.

The redox titrations were plotted on log-linear scales which were convenient for estimating the change of redox poise from a given change of $A_{550-540}$ or $A_{605-630}$.

Estimation of Poise and Apparent Redox Potential of Cytochromes in Transient Mitochondrial States

In experiments where it was not possible to obtain complete redox titrations, the anaerobic mitochondria were first brought to an appropriate point in the redox titration as described in the previous section. The shift of absorbance difference was then recorded as a change of mitochondrial state was induced by adding reagents such as valinomycin or acid or alkali (as indicated in the text and in the legends of the figures), and the shift of apparent midpoint potential was calculated by means of the appropriate redox titration curve.

Results and Discussion

Redox Titrations of Resting Mitochondria

Figure 1 shows redox titrations of *c* and *a* cytochromes in anaerobic rat liver mitochondria equilibrated in a 135 mM KCl medium at pH 7 with valinomycin and FCCP in presence and absence of CO. The curves through the points for cytochromes $(c + c_1)$ and cytochrome *a* (CO present) represent theoretical one-electron transfer titrations, but the experimental curve through the points for cytochromes $(a + a_3)$ (CO absent) corresponds roughly to a one-half-electron transfer process. The presence of CO did not affect the values for cytochromes $(c + c_1)$. Similar titrations over the pH range 5·8 to 7·5 did not differ from those of Fig. 1; and the presence of 1 mM potassium cyanide did not affect the titration curves at pH 7·0. The midpoint potentials for cytochromes $(c + c_1)$ and

cytochrome *a* of 250 mV and 245 mV respectively in the whole mitochondria are comparable with values of 255 mV[27] or 248 mV[28] for cytochrome *c*, 220 mV for cytochrome c_1[28] and 250 mV[20] for cytochrome *a* (CO present) reported for the isolated cytochromes. Caswell[3] obtained a value of the redox potential of cytochrome *a* (cyanide present) of at least 330 mV from a single value of the poise in whole mitochondria, using a potentiometric method for measuring the redox potential of cytochrome *c*, and $A_{445-460}$ to measure the degree of reduction of cytochrome *a*. This value is much higher than our value for cytochrome *a* in presence of CO. It is significant in this context that we found the anomalous redox titration curve of cytochromes $(a + a_3)$, based on $A_{605-630}$, to be unaffected by the presence of cyanide, in agreement with the observations of Minnaert[10] on isolated cytochrome oxidase. The anomalous curve for cytochromes $(a + a_3)$ in the whole mitochondria corresponds closely to that described by Minnaert[10] for the isolated oxidase, and its "midpoint" of 273 mV is 23 mV more positive than that of cytochrome *c* in the intact mitochondria as found by these authors for the isolated cytochromes.

TABLE I. Effect of Donnan or Nernst potential on the apparent midpoint potentials of cytochrome *c* and cytochrome *a*

Medium	E'_m ferricyanide	E'_m cyt. $(c + c_1)$	E'_m cyt. *a*
135 mM KCl	420	255	245
135 mM choline chloride	395	253	231

Rat liver mitochondria (5 mg of protein/ml) were equilibrated in anaerobic, CO-saturated, 135 mM KCl or 135 mM choline chloride medium containing 25 mM sucrose, 3 mM glycylglycine at pH 6·9, 1 μM FCCP, 0·5 μg of valinomycin/ml, 0·5 μg of antimycin A/ml, and 5 mM $Na_4Fe(CN)_6$. The poises of cytochromes $(c + c_1)$ and cytochrome *a* were measured spectrophotometrically during stepwise titration with $K_3Fe(CN)_6$ as described in Materials and Methods.

Tzagoloff and Wharton[20] found a "midpoint" potential of 285 mV for isolated cytochromes $(a + a_3)$. Too much significance should not be attached to the last 5 mV in the absolute redox potential values, and our values for the cytochromes in the whole mitochondria can be regarded as agreeing well with those previously found for the isolated cytochromes.

When the salt used in the suspension medium was 135 mM choline chloride instead of 135 mM KCl, the redox titration curves of the cytochromes were shifted; and this was partly attributable to the dependence of the redox potential of the ferricyanide/ferrocyanide buffer on the cation of the supporting electrolyte.[21] However, when, as shown in Table I, the dependence of midpoint potential of the ferricyanide/ferrocyanide couple on the salt composition of the suspension media was allowed for, the apparent midpoint potential of cytochrome *a* was some 15 mV more negative in the choline chloride than in the KCl medium, but the apparent midpoint potential of cytochromes $(c + c_1)$ in equilibrium with cytochrome *a* was not significantly affected. It was previously shown by Mitchell and Moyle (ref. 17 and supplementary unpublished measurements) that the electric potential and pH difference across the cristae membrane is near zero in anaerobic mitochondrial suspensions equilibrated in presence of FCCP and valinomycin in a 150 mM KCl medium, or in a 135 mM KCl medium containing 5 mM potassium ferro-

cyanide, at pH 7; but that in the corresponding choline chloride medium, or in a medium containing 135 mM choline chloride and 5 mM sodium ferrocyanide, owing to a Donnan or Nernst potential, the inner aqueous phase of the mitochondria is about 0·5 pH unit acid and about 30 mV electronegative relative to the suspension medium. Thus, the apparent midpoint potential of cytochrome *a* is more negative by some 15 mV when the inner phase is some 30 mV more electronegative, or 0·5 pH unit more acid.

Redox Titrations of Mitochondria Hydrolysing ATP

Figure 2 shows redox titrations of the *a* cytochromes corresponding to those of Fig. 1, with and without the addition of ATP, but omitting the valinomycin and FCCP, A in presence of CO, and B in absence of CO. The lower parts of the curves in presence of ATP are not complete because potentials more negative than some +240 mV were not considered to be reliably buffered by the ferricyanide/ferrocyanide system. On adding ATP to the anaerobically equilibrated mitochondria the midpoint potential of cytochrome *a* shifted to an apparent midpoint 40 mV more negative. The tendency of the points in the more oxidizing part of the titration in presence of ATP to come closer to those in absence of ATP was partly attributable to a fall in the rate of ATP hydrolysis and to a corresponding fall in the membrane potential in the latter part of the titration. In the case of the anomalous redox curve of cytochromes $(a + a_3)$, a similar shift of apparent "midpoint" occurred when ATP was added, and there was a reproducible change of shape of the curve as well as a shift of its "midpoint". When the ATPase was inhibited with oligomycin (1 mg/g of mitochondrial protein) there was no shift in the redox curves of the *a* cytochromes when ATP was added to the mitochondrial suspension. Redox titrations of cytochromes $(c + c_1)$ similar to those of Fig. 2 with and without the addition of ATP showed that ATP had no effect on the poise in this case.

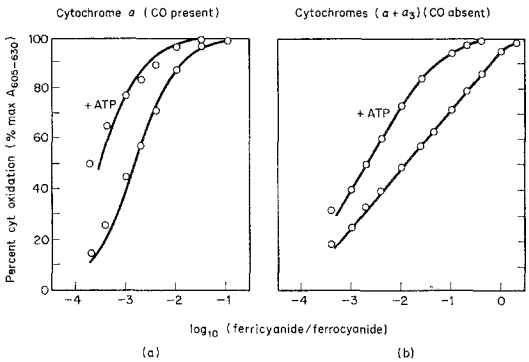

Figure 2. Effect of ATP hydrolysis on the apparent midpoint potential of cytochrome *a* and cytochromes $(a + a_3)$. In A, the mitochondria (5·1 mg of protein/ml) were suspended in anaerobic 135 mM KCl–25 mM sucrose medium saturated with CO and containing 10 mM glycylglycine at pH 7·0, 0·3 μg of antimycin A/ml, and 5 mM $K_4Fe(CN)_6$. In B, the medium was 45 mM choline chloride–190 mM sucrose, CO was absent, and 5 mM $Na_4Fe(CN)_6$ was used, but otherwise the conditions were as in A. For the titration curves marked +ATP, 0·15 mM ATP was added 1 min before beginning the stepwise redox titration with $K_3Fe(CN)_6$. The poises of the cytochromes were measured spectrophotometrically during the redox titration as described in Materials and Methods. The curves in A represent theoretical single-electron transfer titrations.

These observations confirm the finding by Caswell[3] that the addition of ATP to non-respiring mitochondria in presence of antimycin A causes the cytochrome *a* to become oxidized relative to cytochrome *c*. Our observations also show, however, that the change of state of the mitochondria induced by the presence of ATP does not cause any change in the redox potential of cytochromes $(c + c_1)$ relative to the external redox buffer with which the terminal cytochromes are in electronic equilibrium.

Figure 3 shows strip-chart recordings of the change of pK and of $A_{605-630}$ on adding ATP to an equilibrated anaerobic mitochondrial suspension under conditions similar to

those of Fig. 2B, but with valinomycin present to permit K^+ ion equilibration across the cristae membrane and thus to provide the means of calculating the membrane potential. We have omitted the initial part of the pK trace because the base-line is shifted by alkali metal ions present in the ATP solution. The final value of pK after adding FCCP gives the equilibrium pK value. These and other similar results showed that the addition of ATP caused the membrane potential to increase from about 30 mV to about 105 mV and the apparent redox potential of cytochromes $(a + a_3)$ to decrease by about 50 mV, judging from the change of shape and shift of the redox titration curve shown in Fig. 2B. The redox titrations of Fig. 2 were done in absence of valinomycin because more rapid and stable absorbance changes were obtained on adding ATP under these conditions. A slower response of the extinction change in presence of valinomycin was attributable to a slower development of the membrane potential because of the discharging effect of the redistribution of K^+ ions across the membrane.

The membrane potential developed during ATP hydrolysis in absence of valinomycin is probably similar to that developed in presence of valinomycin. Thus, the experiments described in Figs. 2 and 3 indicate that the shift of apparent midpoint potential of cytochrome *a* and of cytochromes $(a + a_3)$ corresponds to about half the change of membrane potential induced by ATP hydrolysis. During ATP hydrolysis in these experiments the inner phase probably became alkaline relative to the outer phase, but no attempt has been made to measure the extent of this change because of the difficulty of correcting for net acid production accompanying ATP hydrolysis.[17, 29]

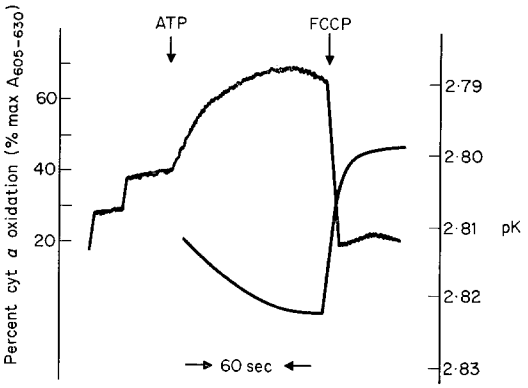

Figure 3. Strip-chart recording of $A_{605-630}$ and pK, indicating redox state of cytochrome *a* and membrane potential development during ATP hydrolysis. The mitochondria (5·0 mg of protein/ml) were suspended in anaerobic 45 mM choline chloride–190 mM sucrose medium saturated with CO and containing 10 mM glycylglycine at pH 7·2, 0·2 μg of valinomycin/ml, 0·3 μg of antimycin A/ml, 0·5 mM EDTA, and 5 mM $Na_4Fe(CN)_6$. After adding 20 μM $K_3Fe(CN)_6$, 0·8 mM ATP and 0·5 μM FCCP were added as shown by the arrows.

Shift of Redox State during K^+ ion Efflux

As shown by Fig. 4, the addition of valinomycin to permit K^+ ion efflux from the mitochondria after equilibrating briefly (2 min) under anaerobic conditions with CO in a medium of low K^+ ion content (0·5 mM), and poising with ferricyanide/ferrocyanide, caused a rapid increase in the degree of oxidation of cytochrome *a*. The subsequent addition of FCCP caused the poise to return to a value near the initial one, and there was a more rapid K^+ ion efflux. Valinomycin has previously been used to cause Ca^{2+} uptake,[30] ATP synthesis,[31] and reversed electron transfer between cytochrome *c* and cytochrome *a*.[3, 32] Since valinomycin is known to cause mitochondrial cristae membranes to become specifically permeable to K^+ ions,[17, 33] the membrane potential in the presence of valinomycin is related to the distribution of K^+ ions across the membrane by the classical Nernst equation (see Materials and Methods) when the conductance of the membrane to the other ionic species present is relatively low. Thus, we have calculated that, in the experi-

ment of Fig. 4, a membrane potential of about 140 mV was developed shortly after the addition of valinomycin, and this decayed to some 125 mV during the following 50 sec. During the period of elevated membrane potential, the apparent midpoint potential of cytochrome *a* fell to 185 mV and then returned towards 190 mV. When FCCP was added, the apparent midpoint potential rose to a value of about 230 mV. In this type of experiment, both EGTA and oligomycin were added in order to decrease electrical "backlash" and to prevent collapse of the membrane potential by reversal of the ATPase.[17] Qualitatively similar results were obtained from $A_{605-630}$ recordings in absence of CO, but these results have not been analysed quantitatively because of the difficulties of interpretation inherent in the change of shape of the redox titration of cytochromes $(a + a_3)$ which is thought to occur (see Fig. 2B). There were no shifts of $A_{550-540}$ corresponding to changes of mitochondrial state induced by valinomycin in this type of experiment in presence or absence of CO, indicating that the apparent midpoint potential of cytochromes $(c + c_1)$ was not affected.

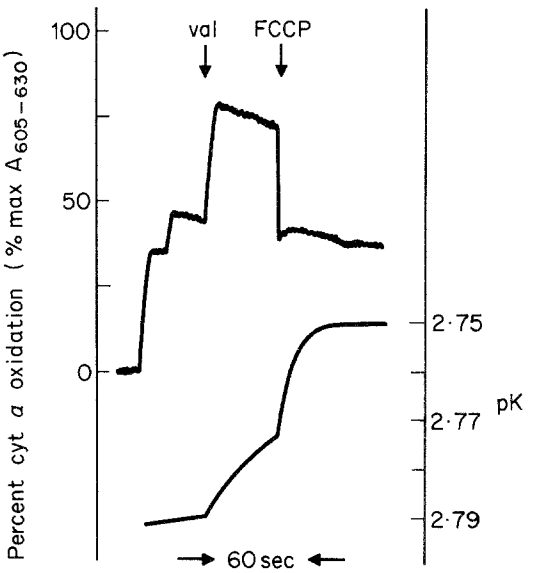

Figure 4. Effect of K^+ efflux, catalysed by valinomycin, on redox state of cytochrome *a*. The mitochondria (5·5 mg of protein/ml) were suspended in anaerobic 45 mM choline chloride–190 mM sucrose medium saturated with CO and containing 3·0 mM glycylglycine at pH 6·8, 0·2 μg of antimycin A/ml, 1 μg of oligomycin/ml, 0·3 mM EGTA, and 5 mM $K_4Fe(CN)_6$. After full reduction of cytochrome *a* was reached, as shown by $A_{605-630}$, 5 μM $K_3Fe(CN)_6$ was added in two steps to poise cytochrome *a* at about 45% oxidized, and valinomycin (0·5 μg/ml) and FCCP (0·5 μM) were added as shown by the arrows. The pK was recorded as described in Materials and Methods.

A series of experiments similar to that of Fig. 4, in which variable proportions of the choline$^+$ in the choline chloride medium was replaced by K^+, gave the dependence of the percentage reduction of cytochrome *a* on the concentration of K^+ ions in the medium, as shown in Fig. 5A. The ferricyanide/ferrocyanide poise was adjusted to give 50% reduction of cytochrome *a* before valinomycin addition. The data of the experiments of Fig. 5A and redox titrations in the same media in absence of valinomycin have been used to calculate the membrane potentials attained after adding valinomycin and the corresponding values of apparent midpoint potential (E''_m) of cytochrome *a*, which are plotted against each other in Fig. 5B. Redox titrations in the choline chloride–KCl media in presence of valinomycin, after the membrane potential due to K^+ ion exit was allowed to subside, did not differ significantly from those in absence of valinomycin; but the mitochondrial suspensions were less stable after the valinomycin treatment and the redox titration values showed relatively more scatter. The line through the experimental points is drawn cutting the vertical axis at a value of apparent midpoint potential E''_m (245 mV), corresponding to the value of the midpoint potential E'_m observed in a medium containing 135 mM KCl and 5 mM potassium ferrocyanide in which the membrane potential is known to be near zero. The slope of the line shows that the displacement of the apparent mid-

point potential corresponds to about half the total electric potential across the cristae membrane.

In the type of experiment described in this section, the inner phase of the cristae became acid relative to the outer phase after valinomycin addition.

Shift of Redox State during H^+ ion Influx or Efflux

Perhaps the most versatile way of varying the membrane potential is to make the cristae membrane specifically permeable to protons with a proton-conducting reagent such as FCCP, and to drive an inward or outward proton current by lowering or raising the pH of the suspension medium.[14] This technique has been used previously to cause K^+ ion uptake in mitochondria in presence of FCCP and valinomycin.[17]

Figure 6 shows the effect of adding acid (outer pH 7·1 to 6·3) and alkali (outer pH 6·3 to 7·1) in a 135 mM KCl medium on the redox poise (curves A–C) of cytochrome *a* and (curves D and E) of cytochromes ($c + c_1$) under various conditions. In presence of FCCP, acid (A) caused rapid reduction and alkali (B) caused rapid oxidation of cytochrome *a*. When valinomycin was present in place of FCCP (C), there was a decrease in the rate of drift of the redox poise after adding acid, but there was no rapid shift of poise. The poise of cytochromes ($c + c_1$) showed no rapid shift on adding acid (D) or alkali (E) in presence of FCCP, but there was a change of the slope of the trace after the pH of the medium was changed. The pH-dependence of the rate of downward drift of the redox poise (towards reduction) was attributable to the fact, established by Estabrook,[26] that the inhibition by antimycin A of the reduction of ferricyanide is more complete at acid than at alkaline pH. Qualitatively similar results were obtained for cytochromes ($a+a_3$), based on $A_{605-630}$ in absence of CO.

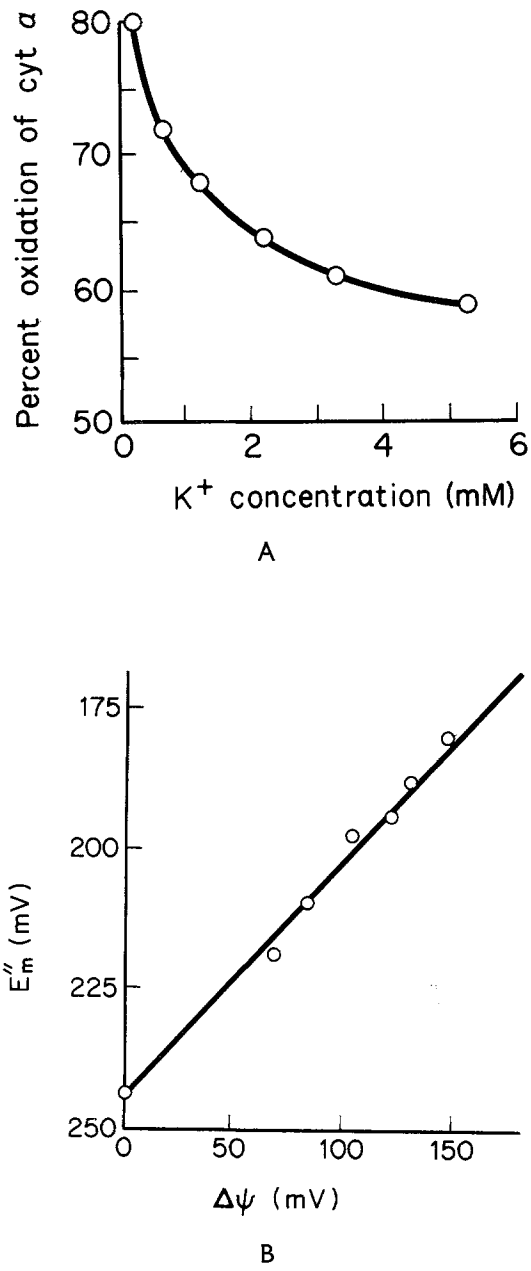

Figure 5. Dependence of percentage oxidation of cytochrome *a* on K^+ concentration in medium during K^+ efflux catalysed by valinomycin (A); and (B) derived relationship between apparent midpoint potential (E''_m) and membrane potential ($\Delta\psi$). Conditions were similar to those of Fig. 4 except that variable proportions of choline$^+$ in the medium were replaced by K^+. Further details in text.

Figure 7 shows the results of a number of experiments like those of Fig. 6A and B in which the amounts of acid and alkali were varied. The membrane potential has been calculated from the pH difference established between the inner and outer phases (see Materials and Methods) and plotted against the change of apparent midpoint potential of cytochrome *a* obtained from the rapid shift of redox poise. The observations show that the change of apparent midpoint potential of cytochrome *a* corresponded to about half the value of the membrane potential, and that this relationship was followed even when the electric polarity across the membrane was the reverse of the physiologically normal polarity.

The rapid absorbance shift observed on adding acid pulses in experiments corresponding to that of Fig. 6A, using a fixed reference wavelength of 630 nm, but varying the measuring wavelength gave the data plotted in Fig. 8. The curve corresponds to the reduced-oxidized absorbance difference spectrum of cytochrome *a* and confirms that the absorbance changes based on $A_{605-630}$ can be taken to give a measure of the redox poise of cytochrome *a* in this type of experiment.

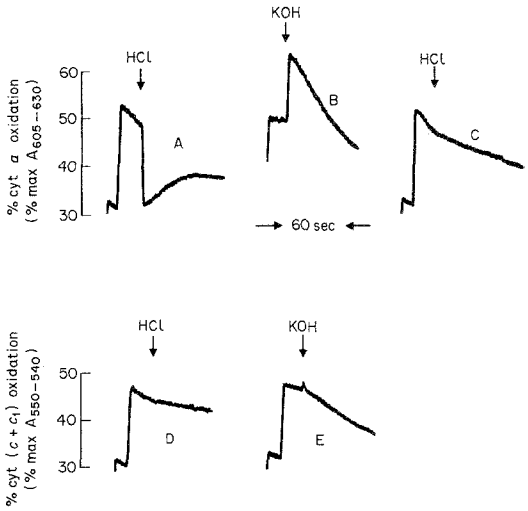

Figure 6. Effects of H^+ influx and efflux in FCCP-treated mitochondria on redox states of cytochromes *a* and $(c + c_1)$. The mitochondria (5·4 mg of protein/ml) were suspended in anaerobic 135 mM KCl–25 mM sucrose medium saturated with CO and containing 3 mM glycylglycine, 0·3 μg of antimycin A/ml, 1 μg of oligomycin/ml, 0·3 mM EGTA, and 5 mM $K_4Fe(CN)_6$. The cytochrome was titrated to 50% oxidation with $K_3Fe(CN)_6$, using the appropriate absorbance difference, and 20 μl of 100 mM HCl or KOH was added so as to shift the outer pH from about pH 7·0 to 6·3 or from pH 6·3 to 7·0, respectively. The additions of HCl and KOH are indicated by the arrows. FCCP (1 μM) was present except in C, where it was replaced by valinomycin (0·2 μg/ml).

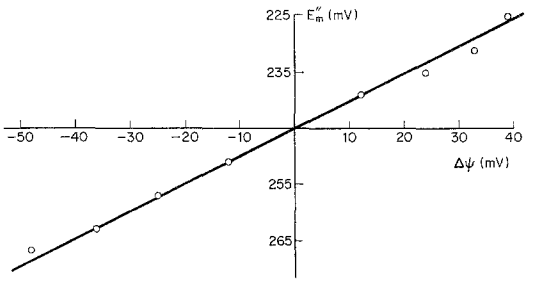

Figure 7. Relationship between apparent midpoint potential (E''_m) and membrane potential ($\Delta\psi$) generated by H^+ influx or efflux. Conditions were as for Fig. 6 except that the mitochondrial suspension contained 5·0 mg of protein/ml.

Possible Relevance of Hypothetical Chemically Coupled Proton Pump or K^+ ion Pump

When the membrane potential is generated by ATP hydrolysis or by K^+ ion efflux catalysed by valinomycin, an inward protonmotive force is generated across the cristae membrane. In this type of mitochondrial energized state, the change of poise of cytochrome *a* relative to cytochromes $(c + c_1)$ might be explained by assuming that there is a chemical "coupling site" between cytochrome *a* and cytochrome *c*, and that cytochrome *a* reacts with a high-energy intermediate or conformational coupling factor that is generated by the reversal of a hypothetical proton pump. This type of system, which has been considered previously by Chappell and Crofts,[34] Slater[35] and Mitchell and Moyle[36] (and see ref. 13), could not, however,

account for the change of poise of cytochrome *a* relative to cytochromes ($c + c_1$) under conditions where there is no protonmotive force across the membrane. It is therefore particularly noteworthy that when the membrane potential is generated by shifting the pH of the outer phase in mitochondrial suspensions treated with FCCP, little or no protonmotive force is generated across the cristae membrane, because the proton-conducting uncoupling agent equilibrates the electrochemical potential of H^+ ions and gives rise to equal and opposite pH and electric potential differences (when expressed in equivalent units) across the membrane. The same condition of electrochemical equilibrium of H^+ ions is also fulfilled in the case of mitochondria equilibrated with valinomycin and FCCP in sucrose media or in choline chloride media of low K^+ ion content when the membrane potential is attributable to a Donnan or Nernst distribution. It follows that the hypothetical chemically coupled proton pump type of mechanism does not provide a general explanation of the observations described in this paper. Similarly, a mechanism involving an electrogenic K^+ ion-pump coupled to an energy-rich intermediate or conformational coupling factor in place of the H^+ ion-pump could not give a general explanation of the results because there can be little or no potassium-motive force when the cristae membrane is made permeable to K^+ ions with valinomycin, as in the experiments of Fig. 3 and Fig. 4.

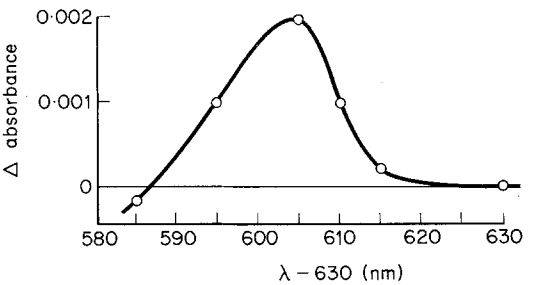

Figure 8. Difference spectrum of component showing change of state when a membrane potential is induced by acid influx. The conditions were as for Fig. 6. Each point represents the change of absorbance difference observed at constant reference wavelength (630 nm) and variable measuring wavelength (λ) between 585 and 630 nm when the pH of the suspension media was suddenly changed from 7·1 to 6·4 by a pulse of 100 mM HCl.

Conclusions

The changes of poise of respiratory carriers that have been observed to accompany changes of energetic state of mitochondrial suspensions[1] have generally been ascribed, in accordance with the chemical or conformational coupling hypotheses, to interactions of energy-rich compounds or complexes with components of the respiratory chain. Such interactions might be the cause of the changes of poise of cytochromes observed in the present work, although certain specific proposals[34-36] along these lines, discussed at the end of the previous section, are not competent to serve as a general explanation of our observations. Our object, however, is to show that the data are susceptible to a particularly simple interpretation in terms of the chemiosmotic coupling hypothesis. This interpretation rests on the fact that the redox potential of an electron carrier is affected by the local electric potential under certain conditions discussed previously[13, 14] and outlined in Materials and Methods. It also depends on the use of FCCP and valinomycin to induce specific permeability to H^+ ions and K^+ ions respectively and thus to allow generation or measurement of the membrane potential.

We have shown that the apparent midpoint potential of cytochromes ($c + c_1$) measured by equilibration with the ferricyanide/ferrocyanide redox buffer in the outer phase is not affected by the various treatments of the mitochondria used in this work to vary the membrane potential. Thus, we conclude that the situation of cytochromes ($c + c_1$) is

probably such that the electric potential varies little in their immediate environment when the electric potential across the cristae membrane changes by some 100 mV. Presumably this implies that cytochromes $(c + c_1)$ are near the surface of or outside the osmotic barrier or M phase of the cristae membrane. This is in conformity with other evidence on the external location of cytochrome *c* recently reviewed by Greville.[37]

The results on the relationship between the apparent midpoint potential (E''_m) of cytochrome *a* and the membrane potential ($\Delta\psi$) are summarized in Table II. The values of $\Delta\psi$ refer to polarity, negative in the inner phase. It is evident that the change of poise of cytochrome *a* relative to and in redox equilibrium with cytochromes $(c + c_1)$ follows the membrane potential for both physiologically normal and abnormal polarities, and that the displacement of E''_m, the apparent midpoint potential, from E'_m, the midpoint potential at zero electric potential, corresponds to about half the membrane potential. This would be expected at electronic equilibrium between cytochrome *a* and cytochromes $(c + c_1)$, as shown in Materials and Methods, if the electric potential in the immediate environment of the electron-accepting groups of cytochrome *a* changed, relative to the

TABLE II. Correlation between membrane potential and displacement of apparent midpoint potential (E''_m) of cytochrome *a* from the midpoint potential (E'_m) defined with respect to zero electric potential

Medium or treatment	$\Delta\psi$	E''_m of cytochrome *a*
135 mM KCl (Fig. 1)		245 mV (defined as E'_m)
135 mM choline chloride (Table I)	About 30 mV	$E'_m - 15$ mV
ATP present (Fig. 2)	About 100 mV	$E'_m - 40$ mV
Valinomycin added (Fig. 5)	Variable positive values	$E'_m - 0{\cdot}43\Delta\psi$
FCCP present, acid or alkali added (Fig. 7)	Variable positive and negative values	$E'_m - 0{\cdot}50\Delta\psi$

outer medium, by about half as much as the membrane potential. Presumably such an environment might correspond to a region in the M phase about mid-way between the surfaces of the outer and inner aqueous phases. Our results indicate only an average distribution of the cytochrome *a* about a mean position, but the fact that the redox titration was not grossly flattened when the membrane potential was induced (by ATP) indicates that the cytochrome *a* could not be dispersed at uniform average concentration through the thickness of the cristae membrane.[13]

The change of shape of the redox titration curve of cytochromes $(a + a_3)$ based on $A_{605-630}$ observed when the membrane potential was induced (Fig. 2B), prompts the speculative suggestion that the haem group of cytochrome a_3 (and the functional copper that may act as intermediary carrier) is located nearer the inner side of the M phase than the haem group of cytochrome *a*.

Acknowledgements

We would like to acknowledge the expert technical assistance of Mr. Robert Harper and Mr. Michael Pearse, and thank Miss Stephanie Phillips and Mr. Roy Mitchell for help in preparing the manuscript and figures. One of us (Peter Hinkle) was the recipient of a Postdoctoral Fellowship (No. 1-F2-GM-22,427-01)

of the National Institutes of Health, General Medical Sciences Division, U.S.A. We acknowledge receipt of a Government Grant for Scientific Investigations, administered by the Royal Society, for the purchase of the Phoenix spectrophotometer used in this work. We are also grateful to Glynn Research Ltd. for general financial support.

References

1. B. Chance and G. R. Williams, *Advan. Enzymol.*, **17** (1956) 65.
2. M. Klingenberg and P. Schollmeyer, *Proc. 5th Internat. Congr. Biochem., Moscow, 1961*, Vol. 5, Pergamon, London, 1963, p. 46.
3. A. H. Caswell, *J. Biol. Chem.*, **243** (1968) 5827.
4. S. Muraoka and E. C. Slater, *Biochim. Biophys. Acta*, **180** (1969) 227.
5. E. C. Slater, *Rev. Pure and Applied Chem.*, **8** (1958) 221.
6. F. Lipmann, in: *Currents in Biochemical Research*, D. E. Green (ed.), Interscience, New York, 1946, p. 137.
7. E. C. Slater, in: *Comprehensive Biochemistry*, Vol. 14, M. Florkin and E. H. Stotz (eds.), Elsevier, Amsterdam 1966, p. 327.
8. R. S. Cockrell, E. J. Harris, and B. C. Pressman, *Biochemistry*, **5** (1966) 2326.
9. E. C. Slater, *FEBS Symposia*, **17** (1970) 205.
10. K. Minnaert, *Biochim. Biophys. Acta*, **110** (1965) 42.
11. T. Horio and J. Ohkawa, *J. Biochem.*, **64** (1968) 393.
12. P. Mitchell, *Biol. Rev.*, **41** (1966) 445.
13. P. Mitchell, *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, Cornwall, 1968.
14. P. Mitchell, in: *Theoretical and Experimental Biophysics*, Vol. 2, Marcel Dekker, New York, 1969, p. 159.
15. P. Mitchell and J. Moyle, *Biochem. J.*, **104** (1967) 588.
16. P. Mitchell and J. Moyle, *Biochem. J.*, **105** (1967) 1147.
17. P. Mitchell and J. Moyle, *European J. Biochem.*, **7** (1969) 471.
18. T. Yonetani, *J. Biol. Chem.*, **235** (1960) 845.
19. D. Keilin and E. F. Hartree, *Proc. Roy. Soc. B.*, **127** (1939) 167.
20. A. Tzagoloff and D. C. Wharton, *J. Biol. Chem.*, **240** (1965) 2628.
21. W. M. Clark, *Oxidation-Reduction Potentials of Organic Systems*, Ballière, Tindall & Cox, London, 1960.
22. P. Mitchell and J. Moyle, *European J. Biochem.*, **9** (1969) 149.
23. B. C. Pressman, *Biochim. Biophys. Acta*, **17** (1955) 273.
24. E. E. Jacobs and D. R. Sanadi, *Biochim. Biophys. Acta*, **38** (1960) 12.
25. R. W. Estabrook, *J. Biol. Chem.*, **236** (1961) 3051.
26. R. W. Estabrook, *Biochim. Biophys. Acta*, **60** (1962) 236.
27. F. F. Rodky and E. G. Ball, *J. Biol. Chem.*, **182** (1950) 17.
28. D. E. Green, J. Järnefelt, and H. D. Tisdale, *Biochim. Biophys. Acta*, **31** (1959) 34.
29. P. Mitchell, *FEBS Symposia*, **17** (1970) 219.
30. G. F. Azzone and A. Azzi, in: *Regulation of Metabolic Processes in Mitochondria*, J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (eds.), Elsevier, Amsterdam, 1966, p. 332.
31. R. S. Cockrell, E. J. Harris, and B. C. Pressman, *Nature*, **215** (1967) 1487.
32. R. S. Cockrell, *Federation Proc.*, **27** (1968) 528.
33. P. J. F. Henderson, J. D. McGivan, and J. B. Chappell, *Biochem. J.*, **111** (1969) 521.
34. J. B. Chappell and A. R. Crofts, *Biochem. J.*, **95** (1965) 393.
35. E. C. Slater, *European J. Biochem.*, **1** (1967) 317.
36. P. Mitchell and J. Moyle, in: *Biochemistry of Mitochondria*, E. C. Slater, Z. Kaniuga, and L. Wojtzcak (eds.), Academic Press, London, 1967, p. 53.
37. G. D. Greville, in: *Current Topics in Bioenergetics*, Vol. 3, D. R. Sanadi (ed.), Academic Press, New York, 1969, p. 1.