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REDOX PROPERTIES OF *b*-TYPE CYTOCHROMES IN *ESCHERICHIA COLI* AND RAT LIVER MITOCHONDRIA AND TECHNIQUES FOR THEIR ANALYSIS

RICHARD W. HENDLER, DAVID W. TOWNE and RICHARD I. SHRAGER*

Section on Cellular Physiology, Laboratory of Biochemistry, National Heart and Lung Institute, Bethesda, Md. 20014 (U.S.A.)

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SUMMARY

We describe here apparatus and procedures for conducting potentiometric titrations and for analyzing the collected data in terms of the number of components present, their amounts and their midpoint potentials. Using these procedures we have determined the presence of three forms of cytochrome b_1 in *Escherichia coli* with midpoint potentials at pH 7.1 of about −50, +110 and +220 mV. We were not able to demonstrate a change in any of these potentials by the addition of phosphate, ATP, or 2,4-dinitrophenol. We have been able to confirm the presence of two forms of cytochrome *b* in non-energized mitochondria and the apparent conversion of the low-potential component to a new high potential component upon energization of the mitochondria. However we cite further experimental data that question the actual conversion of one form of cytochrome *b* to another. An alternative interpretation based on our analysis suggests that the high voltage component may be present in a masked form in the non-energized mitochondria.

INTRODUCTION

The existence of more than one form of cytochrome *b* in mitochondrial electron transport chains has been indicated for many years. (For general reviews see refs 1–4). Observations supporting this conclusion are the following: (1) Antimycin appears to affect a portion of the total cytochrome *b* leading to a red shift of the wavelength of its α absorption by a few nm and to an increase in its state of reduction; (2) the α absorption of cytochrome *b* has maxima at 562, 566 and 558 nm; (3) the addition of ATP enhances the absorption at 566 nm by increasing the state of reduction of one of the suspected *b*-type cytochromes; (4) the addition of O_2 to anaerobic mitochondria in the presence of antimycin and substrate causes a transient partial reduction of

Abbreviation: MOPS, morpholinopropane sulfonic acid.

* Present address: Physical Sciences Laboratory, Division of Computer Research and Technology, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

cytochrome *b*; and (5) the rapid oxidation of reduced cytochrome *b* (in a coupled electron-transport chain) shows a biphasic character with an initial rapid reduction followed by a slower rate of reduction. Recently Wilson, Dutton and their collaborators have introduced a refined technique for potentiometric titration of mitochondria and submitochondrial particles and have shown the existence of two species with different redox properties, one with a midpoint potential of about -30 mV (relative to the hydrogen electrode) and one with a midpoint potential of about +30 mV [5–7]. Evidence that one of these, cytochrome b_t (E_m −30 mV), is a direct participant in the conservation and transduction of energy at phosphorylation site II is the observation that the addition of ATP causes an apparent increase in its midpoint potential of approximately 300 mV (to $E_m \approx +245$ mV). The other, cytochrome b_k ($E_m \approx +30$ mV) is unaffected by the presence of ATP.

We have extensively modified the experimental technique of Dutton and Wilson for obtaining potentiometric data and for the resolution of these data in terms of the number of components present, their percentages, and their midpoint potentials. We have applied these techniques to an analysis of the cytochromes b_1 of the electron transport chain of *Escherichia coli*, both in their particulate forms in the membrane and in their deoxycholate-solubilized forms. We have found three different redox species having E_m values of about −50, +110, and +220 mV. The presence of ATP or dinitrophenol does not appear to influence the amounts or redox properties of these cytochromes.

We have also examined the *b*-type cytochromes of rat liver mitochondria. We are able to reproduce the observations of Wilson et al. [5–7] but point out some problems with the interpretation of these data in terms of an ATP-dependent shift in redox potential of a particular cytochrome *b* species.

MATERIALS AND METHODS

Sources of electron transport chains

E. coli cell envelopes. The total particulate fraction (T) was prepared from penicillin spheroplasts of *E. coli* W6 (ATCC No. 25377) as previously described [8]. The T fraction consists of about 16 % ribosomes and 84 % cell envelopes on the basis of protein content. This material was stored in liquid N_2 at a concentration of about 20 mg protein per ml in a medium containing 21.1 mM Na_2HPO_4, 11 mM KH_2PO_4, 25.6 mM NaCl, 0.39 mM Na_2SO_4, 0.7 mM $MgCl_2$, 25 mM Tris–HCl, and 4.5 % (w/v) glycerol at pH 7.1. In experiments using phosphate buffer, 0.2 vol. of 1 M potassium phosphate, pH 7.1, was added, followed by water to dilute the protein to ½ its original concentration (i.e. to 10 mg/ml). In experiments using morpholinopropane sulfonic acid (MOPS) buffer, 1.5 ml of T fraction was suspended to 12.5 ml in 0.1 M MOPS, pH 7.1, and the envelopes recovered by centrifugation at 105 000 $\times g$ for 60 min. The pellet was suspended to 1.5 ml in a medium containing 25 mM Tris–HCl, 4.5 % (w/v) glycerol, 0.5 mM $MgCl_2$ and 0.1 M MOPS at pH 7.1. Just before use it was diluted 1 : 1 with 0.1 M MOPS.

0.2 % deoxycholate-washed cell envelopes. 6 ml of T fraction were diluted with 6 ml water, 1.2 ml 1 M Tris–HCl, pH 8.1, and 0.26 ml 10 %(w/v) sodium deoxycholate at room temperature. The suspension was centrifuged at 105 000 $\times g$ for 60 min in the cold. The pellet was resuspended in 6 ml of either the phosphate-con-

taining or MOPS-containing buffer described above and either used directly or stored in liquid N_2.

Just before using, the suspension was diluted either with 0.1 M MOPS (pH 7.1) or with 0.2 vol. of 1 M potassium phosphate (pH 7.1) and water to $\frac{1}{2}$ the original protein concentration (i.e. from about 15 mg protein per ml to about 7.5 mg protein per ml).

(1–0.2)% deoxycholate extract of cell envelopes. 1.5 ml of 0.2% deoxycholate washed cell envelopes was thawed and mixed with 0.15 ml of 10% (w/v) deoxycholate. The suspension was centrifuged at $105\,000 \times g$ for 60 min and the optically clear straw-colored supernatant fraction removed. This was diluted either with 0.1 M MOPS or 0.2 vol. of 1 M potassium phosphate buffer plus water to 2 times its original volume, thus reducing the protein concentration from about 5 to 2.5 mg protein per ml. This preparation was used directly.

1% deoxycholate-washed cell envelopes. The pellet from the above extraction was resuspended to 1.5 ml in either the phosphate-containing or MOPS-containing buffer described in the preparation of cell envelopes. The suspension was diluted to $\frac{1}{2}$ its protein concentration with either MOPS or potassium phosphate buffer as described above (i.e. protein diluted from about 10 to 5 mg/ml). This preparation was used directly (without storage).

Rat liver mitochondria. Rat liver mitochondria were freshly prepared according to the procedure of Weinbach [9] and given a final wash in 0.12 M KCl. These mitochondria yield a P/O ratio of 3 with β-hydroxybutyrate or glutamate as substrate and have a respiratory control index of about 8.4. The washed mitochondria were suspended to about 30 mg protein per ml in 0.25 M sucrose and kept on ice. 4 ml of mitochondria were diluted with 5 ml water plus either 1 ml of 1 M K_3PO_4 or 1 M MOPS to give a 0.1 M buffered solution of pH 7.1. Protein concentration was about 12 mg/ml.

Chemicals. Quinhydrone, sodium hydrosulfite ($Na_2S_2O_4$), EDTA, L-ascorbic acid and sodium deoxycholate: Fisher Scientific Co., Fair Lawn, N. J.; potassium ferricyanide: Merck and Co., Inc., Rahway, N. J.; 2-hydroxy-1,4-naphthoquinone: Eastman Organic Chemicals, Rochester, N.Y.; phenazine methosulfate: Calbiochem., La Jolla, Calif.; pyocyanine perchlorate, 1,2-naphthoquinone: K and K Laboratories, Plainview, N.Y.; glycylglycine, MOPS (morpholinopropane sulfonic acid), buffered firefly lantern extract (FLE-250): Sigma Chemical Co., St. Louis, Mo..

Protein was determined by the procedure of Lowry et al. [10] using bovine serum albumin as standard and ATP was determined by the luciferin–luciferase assay of Cole et al. [11].

Argon gas line

Copper tubing (internal diameter 0.032 inch × external diameter 0.086 inch) with 1/8 inch Swage Lok fittings was used throughout. An argon gas cylinder was fitted with a high purity pressure regulator (series 3500 Matheson gas products) containing a stainless steel diaphragm. A control panel with three NRS needle control valves (X-8503) (Brooks Instrument Division, Emerson Electric Co., Hatfield, Pa., 19440) routed the gas either directly through an O_2 analyzer (SKC Inc., P.O. Box 8538, Pittsburgh, Pa., 15220, series 342, calibrated from 0.7 to 100 ppm) or through a scrubbing bottle containing 16 g $Na_2S_2O_4$ in 500 ml 0.2 M Na_2HPO_4, pH 9.25

[12]. After scrubbing, the gas stream was split and sent both to the O_2 analyzer and the reaction vessel. The end of the tubing was inserted into the Teflon cap by means of a right angle-bent 19-gauge hypodermic needle-tube which was soldered to the copper tubing. The O_2 analyzer showed that the level of O_2 in the argon stream was below detectable amounts (i.e. < 0.7 ppm). The flow rate of gas was measured both as it entered the reaction vessel and the O_2 analyzer by means of flow meters fitted with NRS needle control valves (Brooks Instrument Division Model 1355-8506).

Electrodes

Ag/AgCl electrode. A 2-cm length of fine silver wire (99.9 % pure, 0.020 inch diameter) was fastened with lead-free solder into a gold-plated copper connector pin (Relia-Tac, Amphonol Co., Schweber Electronics, Westbury, L.I., N.Y.). The silver wire was prepared for electroplating by 1–2 min exposure first to concentrated NH_4OH and then to 50 % (v/v) concentrated HNO_3. Electroplating was accomplished with a current of < 1 mA over 2–3 h in a solution of 0.05 M HCl. The silver wire was the anode and either a silver or platinum bar served as the cathode. The AgCl-coated electrode had a characteristic plum-colored appearance. Coated electrodes could be stored several days in the dark in a AgCl-saturated solution of 1 mM HCl.

KCl/AgCl agar bridge. Polyethylene tubing (Intramedic PE 200, 0.055 inch $\times$0.075 inch) was filled with a hot 3 % agar in Beckman electrode solution 4787 (4 M KCl saturated with Ag). After the agar was set, the tubing was cut into 5 cm lengths and stored in the Beckman electrode solution.

Pt electrode. A 29 mm $\times$ 3 mm $\times$ 0.25 mm Pt ribbon was heat-impact-welded to a 27-mm length of 0.050 inch Pt wire.

Assembly of measuring chamber (refer to Fig. 1)

The stored Ag/AgCl electrode was rinsed with water and inserted in the hole on top of the Teflon cap diagonally opposite the Pt electrode. An agar KCl bridge tube was placed over the exposed Ag/AgCl electrode and inserted into the depression on the bottom of the cap. The Ag/AgCl reference electrode was connected to a pH meter (Radiometer Model PHM4C) by means of a Teflon-insulated, 30-gauge, stranded wire fitted with a (Relia-Tac) female connector. The voltage of the micro Ag/AgCl reference electrode was checked against a Beckman No. 1170-71 Calomel electrode ($E_h = +246$ mV), a Beckman 39186 Ag/AgCl electrode ($E_h = +200$ mV), and a Leeds and Northrop miniature 124 138 Ag/AgCl electrode assembly in 2 M KCl ($E_h = +219$ mV). The potentials of all reference electrodes including the newly constructed micro Ag/AgCl when assayed in all combinations were found to be correct to within ± 3 mV. The Pt electrode was washed in 50 % concentrated HNO_3 before use. It was occasionally checked against other Pt or reference electrodes. The argon gas line was then attached to the cap through the remaining opening diagonally opposite the vent tube. An appropriate length of plastic tubing was fitted to the metal gas flushing line to extend its length to the bottom of a standard (1 cm $\times$ 1 cm $\times$ 4.5 cm) optical cuvette and a small amount of Dow Corning antifoam AF was placed at the top of the bubbling tube or on the top inner surface of the cuvette which contained 3 ml of sample to be analyzed. The cap assembly was inserted into the cuvette and a strip of parafilm was used to seal the interface between cap and cuvette. The entire assembly was placed in the sample compartment of an Aminco Chance

APPARATUS FOR POTENTIOMETRIC TITRATIONS

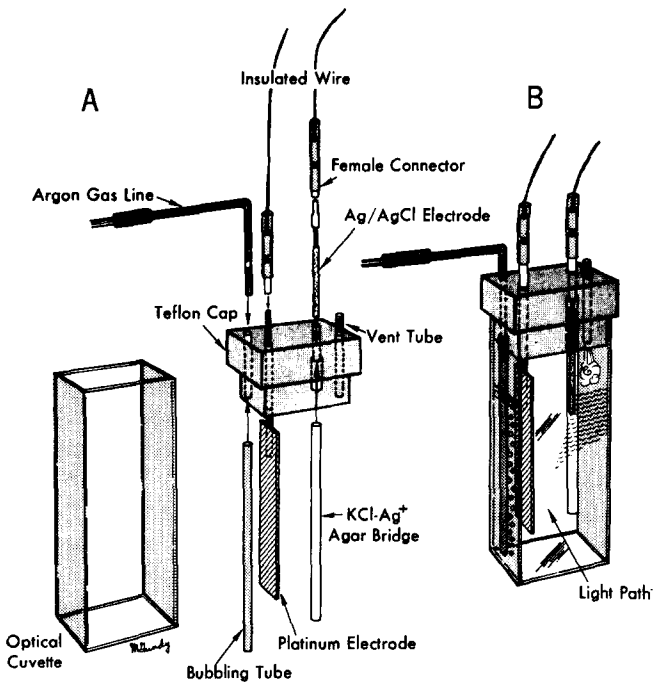

Fig. 1. The components used for assembly of the potentiometric titration chamber are shown in Part A. The assembled apparatus is shown in Part B. Further details for the assembly and use of the chamber are described in the text.

dual wavelength/split beam spectrophotometer (American Instrument Co., Silver Spring, Md.).

PROCEDURE

Anaerobiosis was established by endogenous respiration plus bubbling with argon for 20 mins. The following five mediators were then added either separately or in a mix: quinhydrone ($E'_m = +280$ mV), 1,2 naphthoquinone ($E'_m = +143$ mV), phenazine methosulfate ($E'_m = +80$ mV), pyocyanine ($E'_m = -34$ mV) and 2-hydroxy-1,4-naphthoquinone ($E'_m = -145$ mV). The same results were obtained with mediator concentrations from 10 to 60 μM and in experiments where 1,2-naphthoquinone was omitted. Lack of mediation was indicated by either the absorbance or voltage changing independently of the other. Mediation was indicated by the following: (1) the addition of more mediators did not change either the absorbance or voltage; (2) an absorbance increase was accompanied by a decrease in voltage of the reaction vessel contents and vice-versa; (3) the same values for extent of reduction and voltage of the reaction medium were reproduced in successive titrations and (4) in repeat experiments done on different days, the same E_m values were reproduced. Mediators were added during the experiment whenever indications of inadequate

mediation were encountered. Reduction was accomplished with endogenous substrates and traces of 0.4 M ascorbic acid in the range of about +450 to +50 mV, with 0.4 M NADH from about +50 to −50 mV, and with 0.2 M $Na_2S_2O_4$ in 0.2 M Na_2HPO_4 from about −50 to −150 mV. Oxidation was accomplished with traces (< 1–4 μl) of 0.8 M potassium ferricyanide. Titration curves were checked by either successive reductions spaced with ferricyanide oxidation or by a reductive cycle followed by an oxidative cycle. A single reductive or oxidative titration usually yielded 50–100 pairs of voltage (E) and extent of reduction (R) values and took about 1.5 h. Extent of reduction for cytochrome b_1 was measured by dual-wavelength spectrophotometry at the peak (560 nm) and isosbestic point (550 nm) [13] at room temperature. For cytochrome b, the wavelength pair 561.5 and 575 nm was used [7]. It was determined that neither mediators nor ferricyanide interfered with the measurements at these wavelengths.

Analysis of data

Theory [*14*]. The relation between the electrical potential of a medium and the ratio of oxidized to reduced forms of any oxidizable component is:

$$E_h = E_m + (RT/nF) ln [ox]/[red] \quad (1)$$

where E_h is the electrical potential of the medium relative to the hydrogen electrode; E_m is the midpoint potential (i.e. where [ox] = [red]) at the pH stated; R is the universal gas constant, F is Faraday's constant; T is the absolute temperature and n is the number of electrons transferred from reduced to oxidized forms. For a one-electron transfer such as expected during the oxidation or reduction of individual cytochromes, and for the reactions at 23 °C with all potentials expressed in millivolts, the expression is simplified to:

$$E_h = E_m + 59 \log_{10} [ox]/[red] \quad (2)$$

Therefore, for a single component, a plot of E_h vs log [ox]/[red] should result in a straight line with a slope of 59 and an E_h intercept at the value of E_m. Two or more components, each obeying the same general relationship, lead to a composite curve having a sigmoidal character. If the data is plotted as R (amount reduced) vs E (i.e. E_h) a curve analogous to a pH titration curve is obtained, where amount neutralized is plotted against pH. Inflection points occur at E_m values in the redox curves just as at pK values in pH titration curves. A sigmoidal step occurs in the curve for each of the components present. When components have close E_m or pK values, or are present at greatly different concentrations the composite curves are not resolvable by eye.

The data of the experiments are voltages and corresponding absorbances which represent the cumulative state of reduction for the species of cytochrome b_1 (or b) present. The object of the analysis is to resolve the data in terms of number of components present, the amount of each and the value of each midpoint potential.

Data processing. The accumulated data was analyzed by MLAB, a mathematical modeling system programmed for the DEC system 10. The MLAB system responds to a curve-fitting command after the user supplies a data matrix, an analytic function and a list of parameters that govern the shape of the function. MLAB then adjusts the parameters to make the function fit the last column (dependent variable)

of the data matrix. Error estimates and dependency values are produced for judgements about resolution and possible over parameterization [15, 16].

In the beginning, we tried fitting the data in two ways. One employed the relation of E to log[ox]/[red], because this has been the most popular graphic display form in the literature. The other, fit the amount reduced (R) as a function of E. Both approaches will be described, although, after the first several experiments it was decided that the latter method was superior to the former.

Definition of functions used

$R[E]$: Function R of E is derived by solving Eqn 2 for R([red]).

$\mathrm{R}[E] = T/(1+10^{(E-E_m)/59})$ where T = total amount of reducible material. For a mixture of several components, each component contributes its own R[E] value. For example, for a two component mixture the function RF2[E] is used:

$$\mathrm{RF2}[E] = \frac{T1}{1+10^{(E-E_{1m})/59}} + \frac{T2}{1+10^{(E-E_{2m})/59}}$$

In order to express the log [ox]/[red] relationship, L[R] is defined:

$$\mathrm{L}[R] = \log [\mathrm{ox}]/[\mathrm{red}] = \log\left(\frac{T}{R_{\mathrm{obs}}} - 1\right)$$

Where R_{obs} = the observed amount of reduction. The log [ox]/[red]value in terms of E is:

$$\mathrm{LE}[E] = \log\left(\frac{T}{\mathrm{R}[E]} - 1\right)$$

For two components, this becomes:

$$\mathrm{LF2}[E] = \log\left(\frac{T1+T2}{\mathrm{RF2}[E]} - 1\right)$$

Two other important functions which will be discussed below are defined as LDF [E, R] = L[R]−LE[E] and RD[E] = R[E]−D. D is a correction factor described below.

Curve-fitting procedure

A two-column matrix is formed from the data. The first column contains the independent variable, E, and the second column contains the observed dependent variable. The computer is asked to fit the parameters $T1$, $T2$, . . . TN and E_{m1}, E_{m2} . . . E_{mN} to a function of E so that the computed value for the dependent variable is the closest to the observed value in column 2 of the data matrix. In order to fit the data to a log[ox]/[red] relationship the R_{obs} values must be converted to log values by using the L[R] function. This however, forces us to assign a value to T rather than allowing this value to be fitted by the computer. To avoid this problem, the function LDF[E,R] was used and was fit to a vector of zeroes. The computer was asked to vary the parameters T and E_m so that the log [ox]/[red] computed from R_{obs} was close to that computed from R[E]. A three column data matrix was used in this case and was composed of two columns of independent variables (E and R) and a column of zeroes as the dependent variable.

Fitting the data to R_{obs} is more direct and the two-column matrix consists simply of E values (the independent variable) and R_{obs} (the dependent variable). An additional parameter, D, was added to remove any uncertainty as to the true value for the baseline where $R = 0$. This is important because all R values recorded have an absolute value relative to a zero value which is subject to experimental uncertainty. The parameter, D, is simply a correction which is added to all values of R_{obs} by the fitting routine. The function used for this fit is RD[E].

The output from the computer contains the fitted values for the amount of each species ($T1, T2 \ldots TN$), the corresponding midpoint potential ($E_{m1}, E_{m2} \ldots E_{mN}$) and the baseline correction, D. Standard errors and dependency values are given for each fitted parameter. The dependency value indicates the extent to which the parameter is dependent on the values of the other parameters. The closeness of the fit is indicated by the root mean square deviation (R.M.S.) for the average observed dependent variable (i.e. extent of reduction). When the curve fitting procedure developed values for parameters that were extremely large (e.g. $> 10^{20}$), the procedure was interrupted with a message printed at the terminal. This could occur if more components are specified than are actually present. The computer also has the capacity to control an accessory graphic display plotter (such as a Calcomp model 663, California Computer Co., Anaheim, Calif.; or a Zeta series 230, Zeta Research Inc., Lafayette, Calif.). The graphs displayed in Figs 2–8 were drawn by a Calcomp plotter.

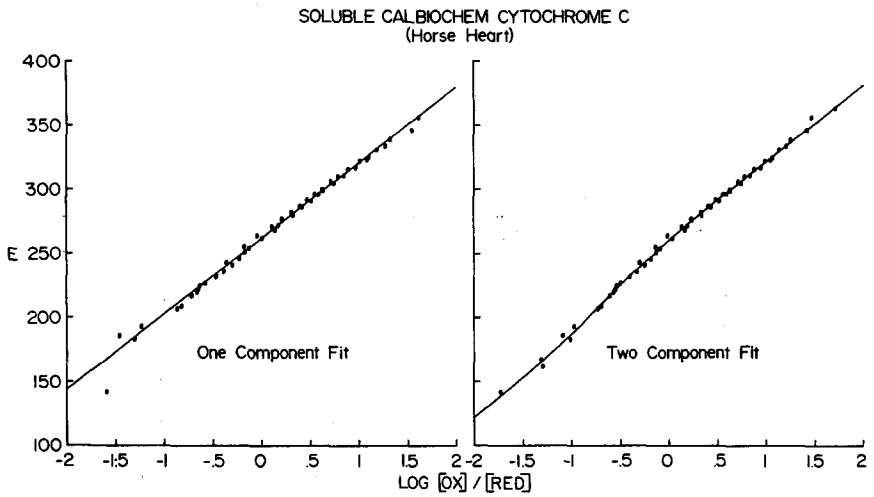

Fig. 2. The solution analyzed (3 ml) contained 0.82 mg/ml soluble cytochrome c in 0.1 M potassium phosphate at pH 7.0 plus 23 μM each of phenazine methosulfate (PMS) and quinhydrone. Absorbance was determined as the difference in absorbance between 550 and 540 nm. The starting voltage was adjusted to $\pm$380 mV with 3 μl of 80 mM $K_3Fe(CN)_6$. Reduction was accomplished with 0.5–1.5 μl additions of 4 mM ascorbate. After collecting 22 pairs of voltage and corresponding absorbance data covering the range of +380 to +130 mV, an oxidative cycle was accomplished with 0.5–3 μl additions of 8 mM $K_3Fe(CN)_6$. This stepwise oxidation yielded 31 pairs of voltage and absorbance measurements covering the range of +130 to +387 mV. The figure shows the combined data converted to log (oxidized/reduced) (i.e. log $(T/R-1)$) in the form of small circles. The solid line is the least squares best fit derived from a computer analysis for one component or two components. The ordinate is calibrated in mV (E) relative to the hydrogen electrode. The root mean square error is 1.61 % for the one component analysis and 1.23 % for the two-component analysis.

Test application

Cytochrome *c* (Calbiochem, equine heart cytochrome *c*) was analyzed using the wavelength pair of 550 and 540 nm to determine extent of reduction by dual wavelength measurements. Fig. 2 shows the results of an RD[*E*] fit of the data for one and two components depicted in a plot of *E* vs log [ox]/[red]. Although the observed points are very close to the one component derived straight line with a slope of 59 and an E_m value of +262 mV, a better fit (i.e. R.M.S. of 1.23 % compared to 1.61 %) was obtained for a two component system with the major component comprising 87.8 % and having an E_m value of +266 mV, characteristic of cytochrome *c* [5] and the minor component comprising 12.2 % and an E_m value of +195 mV, close to the +215 mV reported for cytochrome c_1 [17]. The same data expressed as %*R* vs *E* is shown in Fig. 3.

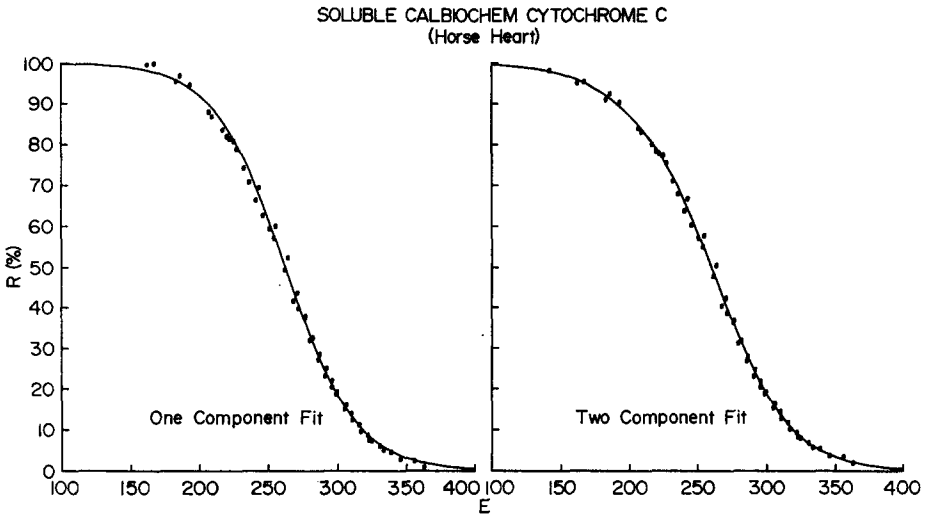

Fig. 3. The same data used in deriving Fig. 2 is represented here in a plot of percent reduced (*R*(%)) vs. *E* in mV. Refer to Fig. 2 for further details.

It should be mentioned at this time that the same data represented as either a two or three component fit will appear different. This is because the computed total in a two component solution ($T1+T2$) will most likely be different than that in a three-component solution ($T1+T2+T3$). This is also true for the baseline correction, *D*. These differences are reflected in the plotted values for percent of reduction or log [ox]/[red] calculated from the observed *R* values.

RESULTS

Experiments with E. coli

Number of cytochrome b_1 components. Fig. 4 shows a plot of *E* vs log [ox]/[red]. Because the points do not fall on a straight line, more than a single species are present. Fig. 4 also shows the derived fits for two- and three-component mixtures. Fig. 5 shows the experimental data and derived fits for two and three component mixtures

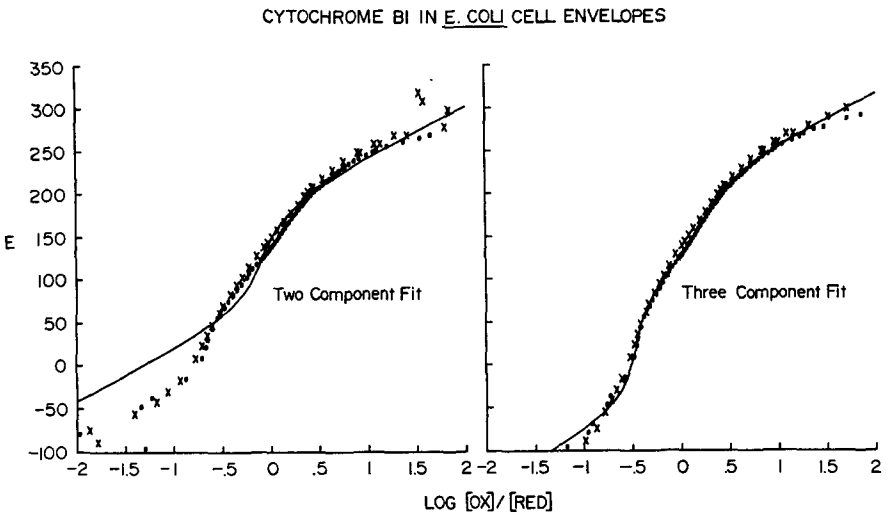

Fig. 4. The suspension analyzed (3 ml) contained *E. coli* cell envelopes (10 mg protein per ml), 105 mM potassium phosphate, 10 mM sodium phosphate, 13 mM NaCl, 0.2 mM Na_2SO_4, 0.35 mM $MgCl_2$, 12.5 mM Tris–HCl, and 2.2 % (w/v) glycerol at pH 7.1. Mediators used in this experiment were 46 μM each of quinhydrone, phenazine methosulfate, pyocyanine, and 2-hydroxy-1,4-naphthoquinone. Absorbance was determined as the difference in absorbance between 560 and 550 nm. The starting voltage was adjusted to +360 mV with 1 μl of 0.8 M $K_3Fe(CN)_6$. Reduction was accomplished with electrons from endogenous substrate and traces of 40 mM ascorbate and 0.4 M NADH. After collecting 75 pairs of voltage and absorbance data covering the range of +300 to −134 mV, an oxidative cycle was performed with the addition of traces of 0.8 M $K_3Fe(CN)_6$. An additional 45 pairs of voltage and absorbance data covering the voltage range −134 to +315 mV was then collected. The figure shows the voltage, E, of the medium in mV vs the collected absorbance data converted to log (oxidized/reduced) (i.e. log $T/R-1$)). The data representing the reductive cycle is shown as open circles and the data representing the oxidative cycle as "X". The solid line shows the least squares best fit for two or three components. The computer analysis was performed on all 120 pairs of collected data and the root mean square error for computed and observed "percent reduced" was 4.33 % for the two-component analysis and 1.36 % for the three-component analysis.

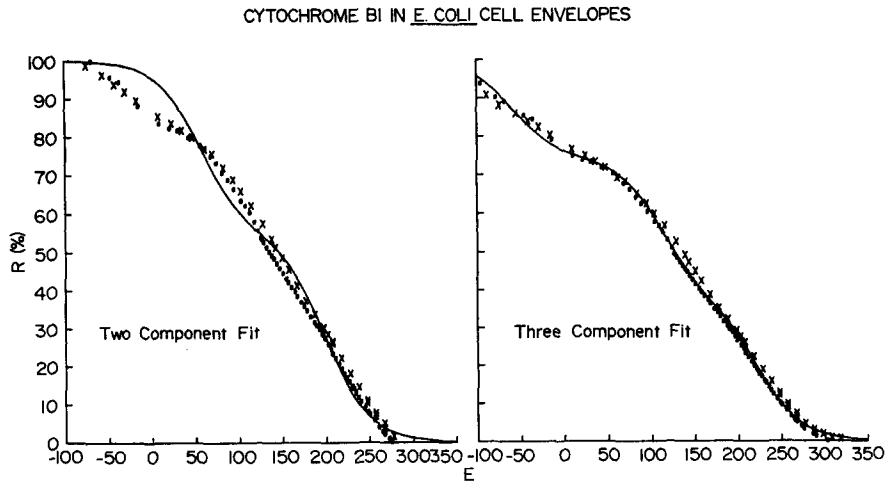

Fig. 5. The same data used in deriving Fig. 4 is represented here in a plot of percent reduced (R(%)) vs E in mV. Refer to Fig. 4 for further details.

in a plot of $\%R$ vs E. The data are better represented by the three-component solution.

Data from all experiments were consistently fit to functions for 2, 3, 4, and 5 components. The results suggest that three components are present. When 4- and 5-component fits were tried, either of two results occurred. In the one case, two of the derived E_m values centered on a single value, and displayed high dependency values indicating there was really no discernable difference between them. This then reduced the actual fit from 4 or 5 back to 3 components. In the other case, one of the original 3 E_m values obtained in a 3-component fit split into two components with E_m values on either side of the original value. When this happened, the standard errors for each of the 2 new E_m values were considerably larger than that of the single parent E_m value. An additional argument for 3 components, rather than 4 or 5 is that the same 3 values persistently appear in all four types of preparation analyzed (discussed below). This consistency did not obtain for the 4- and 5-component fits.

Analyses were performed on cytochrome b_1 present in intact cell envelopes, 0.2 % deoxycholate-washed cell envelopes, a 1 % deoxycholate-soluble extract of the 0.2 % deoxycholate-washed envelopes, and the remaining 1 % deoxycholate-extracted cell envelopes. The purpose of the 0.2 % deoxycholate wash was to lower the contribution of reducing power from endogenous substrates. This wash also removes a portion of endogenous membrane associated dehydrogenases [8]. The 1 % deoxycholate solubilizes about half of the cytochrome b_1, the remainder being tightly associated with the residue from this wash [8]. If we assume that the same three components are present in all four of these preparations, we obtain the average midpoint potentials and standard errors shown in Part 1 of Table I for 14 experiments. It is also seen that the same 3 components are present in 8 experiments done in the presence of added 6 mM ATP and in 8 experiments in the presence of 1 mM 2,4-dinitrophenol. Part 2 shows the data collected for cell envelopes alone and includes percentages for each of the three components. The E_m values are not apparently different from the overall averages shown in Part 1. There was no noticeable effect caused by the presence of ATP or 2,4-dinitrophenol. The same comments are essentially applicable for the Em values derived from analysis of the 0.2 % deoxycholate-washed envelopes (Part 3), the 1 % deoxycholate-soluble cytochrome b_1 (Part 4), and the 1 % deoxycholate-extracted envelopes (Part 5). If the total cytochrome b_1 is taken as that present in both the 1 % deoxycholate extract and its residue, the percentage of each component shown in Parts 4 and 5 is obtained. Distribution and recovery of cytochromes based on the content of the parent fraction (0.2 % deoxycholate-washed envelopes, Part 3) was as follows. For the high-potential component, 10 % was found in the soluble portion and 56 % in the residue. For the middle potential component, about 45 % was found in each of the soluble and insoluble fractions. All of the recovered low potential component (41 %) was found in the soluble fraction. Overall recovery of cytochrome was about 60 %, distributed equally between the two fractions.

As emphasized by Wilson et al. [1] for a reaction involving ATP such as: L+cytochrome b_1+ATP → cytochrome b_1 ~ L+ADP+P the equilibrium concentration of cytochrome b_1 ~ L would depend on the phosphate potential (i.e. ratio [ATP]/[ADP][P]): cytochrome b_1 ~ L = (K_{eq} [L] [cytochrome b_1]) ([ATP]/[ADP][P]).

Although 6 mM ATP and no ADP was added, a low amount of ATPase activity may generate enough ADP so that in the presence of the phosphate buffer,

TABLE I

CYTOCHROME b_1 COMPONENTS OF *E. COLI* (PHOSPHATE BUFFER)

Values in the table represent averages ±S.E. for the number of experiments listed. Protein concentrations for cell envelopes, 0.2 % deoxycholate-washed cell envelopes, (1–0.2)% deoxycholate-soluble extract and the 1 % deoxycholate-washed cell envelopes respectively were about 10, 7.5, 2.5, and 5 mg/ml. Corresponding total absorbances (560 minus 550 nm) for fully reduced cytochromes b_1 were 0.085, 0.071, 0.019, and 0.022. The cell fraction was suspended in 3 ml of a medium containing 105 mM potassium phosphate, 10 mM sodium phosphate, 13 mM NaCl, 0.2 mM Na_2SO_4, 0.35 mM $MgCl_2$, 12.5 mM Tris–HCl, and 2.2 % (w/v) glycerol at pH 7.1. ATP when present was added at 6 mM and 2,4-dinitrophenol at 1 mM. Redox mediators were added and potentiometric titrations performed as described in the text and in the legend to Fig. 4. The percentage of each component ($T1$, $T2$, and $T3$) and the corresponding midpoint potentials (E_{m1}, E_{m2}, and E_{m3}) were determined by computer analysis based on non-linear regression analysis as described in the text.

Source	Number of experiments	Percent of each component			Midpoint potentials		
		$T1$	$T2$	$T3$	E_{m1}	E_{m2}	E_{m3}
1. All listed below							
No additions	14				222±5.3	107±3.7	−47±5.1
+ATP	8				221±7.0	119±5.8	−52±9.7
+DNP	8				213±6.8	113±7.0	−38±7.8
2. Cell envelopes							
No additions	4	39.0±1.2	33.4±1.9	27.6±1.1	232±8.0	99±93	−47±6.5
+ATP	3	38.5±1.2	30.7±2.5	30.8±2.1	238±9.9	120±14.4	−34±5.6
+DNP	3	34.7±2.5	33.2±4.3	33.1±1.9	219±2.1	112±5.3	−38±6.5
3. 0.2 % deoxycholate-washed cell envelopes							
No additions	4	36.3±1.3	42.2±2.5	21.5±3.9	222±4.7	106±6.5	−44±16
+ATP	2	38.9±2.8	28.9±3.4	32.2±0.6	202±10.5	122±16	−68±20
+DNP	3	33.4±2.6	42.8±0.6	25.9±4	216±19	116±20	−38±18
4. (1–0.2)% deoxycholate-soluble extract							
No additions	2	5.2±1.6	26.9±0.2	19.3±2.8	218±11	109±5	−54±3
+ATP	1	6.4	19.4	23.2	209	110	−73
5. 1 % deoxycholate-washed cell envelopes							
No additions	3	29.0±2.5	23.7±4.3		209±17	114±1.8	
+ATP	2	26.9±2.1	30.9±4.6		223±9.0	119±1.0	
+DNP	2	27.9±5.4	21.8±11		201±5.0	110±4.0	

TABLE II

CYTOCHROME b_1 COMPONENTS OF *E. COLI* (MOPS BUFFER)

The legend to Table I applies also to the data in this table except for the composition of the suspending medium. The cell fractions were suspended in 3 ml of a medium containing 100 mM MOPS, 0.25 mM $MgCl_2$, 12.5 mM Tris–HCl, and 2.2 % (w/v) glycerol at pH 7.1.

Source	Number of experiments	Percent of each component			Midpoint potentials		
		*T*1	*T*2	*T*3	E_{m1}	E_{m2}	E_{m3}
1. All listed below							
No additions	10				223±5.4	104±5.8	−60±11.9
+ATP	13				223±5.5	107±3	−68±9.6
2. Cell envelopes							
No additions	2	40±1.7	34±2.0	26±0.3	238±13	103±3.5	−57±20
+ATP	2	38±0.4	33±2.3	29±2.0	240±5	107±6	−63±3
3. 0.2 % deoxycholate-washed cell envelopes							
No additions	2	43±2.5	41±1.4	16±1.1	211±6.0	104±3.5	−70±23
+ATP	6	40±2.9	38±0.9	22±2.7	213±4.3	100±2.7	−60±14
4. (1–0.2)% deoxycholate-soluble fraction							
No additions	3		37±5.2	14±2.0		119±7	−56±25
+ATP	2		25±2.9	20±3.6		108±8	−96±14
5. 1 % deoxycholate-washed cell envelopes							
No additions	3	27±7.1	22±8.1		221±4.7	91±13	
+ATP	3	28±1.5	24±3.8		232±14	119±3.5	

the amount of cytochrome $b_1 \sim$ L present would be below detectable levels. Having established the E_m values for the non-energized system as shown in Table I, we repeated the experiments with MOPS buffer replacing the phosphate. The data in Table II show essentially the same picture as was found in the experiments where 100 mM phosphate was present. The addition of ATP did not cause a dramatic change in the relative amounts or midpoint potentials of the indicated cytochrome b_1 components.

Experiments with rat liver mitochondria

Two cytochromes *b* have been reported to be present in rat liver mitochondria. One, cytochrome b_k, has an E_m of about 30 mV and the other, cytochrome b_t has an E_m of about −30 mV [1, 5]. We also find these two components in non-energized mitochondria using either phosphate or MOPS buffer (Table III). Wilson et al. report that in energized mitochondria, the low-potential species is absent and a new species of high potential is present. Wilson et al. conclude that the low-potential component (energy-transducing cytochrome b_t) has been transformed by ATP into a new high-potential form. The results of our two-component solutions (Table III) confirm the findings of Wilson et al. We tried fitting the same data to 3 components. The non-energized system either produced values for the parameters outside the permitted range or yielded a 3-component solution which was less satisfactory because of greater uncertainty in the fitted parameters. However, in the case of energized mitochondria, the 3-component fit was always superior to the 2-component fit (Table III, Figs 6 and 7). The 3-component solution for energized mitochondria leads to a

TABLE III

CYTOCHROME *b* COMPONENTS OF RAT LIVER MITOCHONDRIA

Values in the table represent averages ±S.E. for the number of experiments listed. Mitochondria were present at a concentration of 12 mg/ml in a medium containing 5 μM rotenone, 100 mM sucrose and either 100 mM potassium phosphate or MOPS buffer at pH 7.1. Both "non-energized" and "energized" mitochondria were obtained under a variety of different circumstances as described in the text. Total absorbances (561.5 minus 575 nm) for fully reduced cytochromes *b* were 0.050, 0.0485, 0.059, and 0.0715 respectively for "non-energized" mitochondria in phosphate and in MOPS buffers and for 2- and 3-component analyses for "energized" mitochondria in MOPS buffer. Redox mediators were added and potentiometric titrations performed as described in the text and in the legend to Fig. 6. The percentage of each component ($T1$, $T2$, and $T3$) and the corresponding midpoint potential (E_{m1}, E_{m2}, and E_{m3}) were determined by computer analysis based on non-linear regression analysis as described in the text. R.M.S., root mean square deviation.

	Non-energized*		Energized* (in MOPS)	
	In phosphate	In MOPS	2 components	3 components
% $T1$	35±5.4	35±7.9	45±3.4	32±4.0
% $T2$	65±5.4	65±7.9	55±3.4	38±2.4
% $T3$	—	—	—	30±3.5
E_{m1}	61±4.7	64±6.1	259±13	263±9
E_{m2}	−28±14	−9±5.5	65±14	96±8.8
E_{m3}	—	—	—	−20±7.7
Number of experiments	5	8	5	7
R.M.S.	1.63±0.20	1.69±0.3	2.73±0.16	1.13±0.17

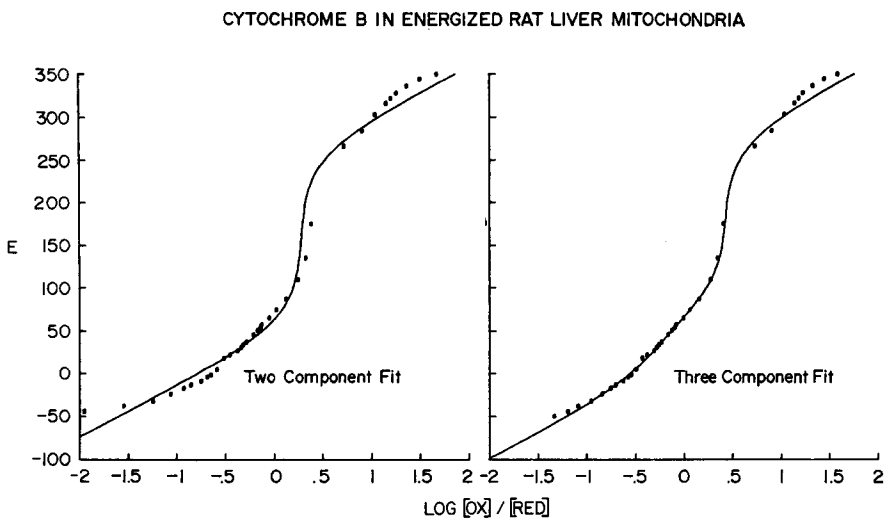

Fig. 6. The suspension analyzed (3 ml) contained rat liver mitochondria (12 mg protein per ml), 5 μM rotenone, 100 mM sucrose and 100 mM MOPS buffer at pH 7.1. Mediators used in this experiment were 32 μM each of quinhydrone, 1,2-naphthoquinone, phenazine methosulfate, pyocyanine, and 2-hydroxy-1,4-naphthoquinone. Absorbance was determined as the difference in absorbance between 561.5 and 575 nm. The starting voltage was adjusted to +384 mV with 6 μl of 0.8 M $K_3Fe(CN)_6$. ATP (6 mM) was added at a voltage of +374 mV and a fast spontaneous reduction by endogenous substrates lowered the voltage to 0 mV in 6 min. A slower rate of reduction by endogenous substrate brought the voltage to −71 mV. The voltage was then raised with $K_3Fe(CN)_6$; 16 μM each of all mediators were added, and a repeat titration started at 26 min after the addition of ATP. The mitochondria now behaved as "non-energized" (see text). The second titration is shown in Fig. 8. 46 pairs of voltage and absorbance data collected during the first titration (immediately following ATP addition) were used for the curve-fitting analysis represented in this figure and Fig. 7. Voltage E (mV) is plotted vs extent of reduction, expressed as log (oxidized/reduced) (i.e. log $(T/R-1)$). Actual data is shown as open circles and the computed best fits for two components and for three components are shown as solid lines. The root mean square error for computed and observed "percent reduced" was 2.75 % for the two-component analysis and 1.13 % for the three-component analysis.

different conclusion than was indicated by the 2-component solution. First of all, a low potential species remains (-9 ± 5.5 mV non-energized, -20 ± 7.7 mV energized) and the high-potential species emerges as a newly seen component. The total absorbances in the non-energized and energized cases were 0.049 and 0.0715 respectively. The additional absorbance obtained in the energized case was entirely accounted for by the high-voltage component. This is additional evidence that the high-voltage component may not be formed by transformation of a preexisting low-voltage component.

In our experience, we have recognized some troublesome experimental problems with mitochondria which were not present with *E. coli* cell envelopes. With non-energized mitochondria, in the voltage range of about +350 to +150 mV, there was an anomalous decrease in optical absorption accompanying a decrease in voltage (Fig. 8). We therefore took for analysis only data collected for voltages < 150 mV. However, the anomaly obscures information on the possible presence of a high-potential species and probably affects the values derived from lower voltage data.

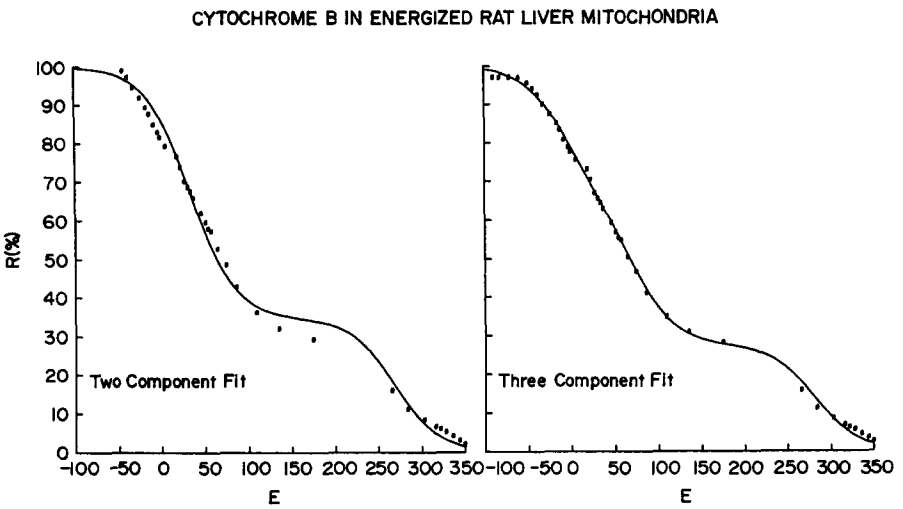

Fig. 7. The same data used in deriving Fig. 6 is represented here in a plot of percent reduced ($R(\%)$) vs E in mV. Refer to Fig. 6 for further details.

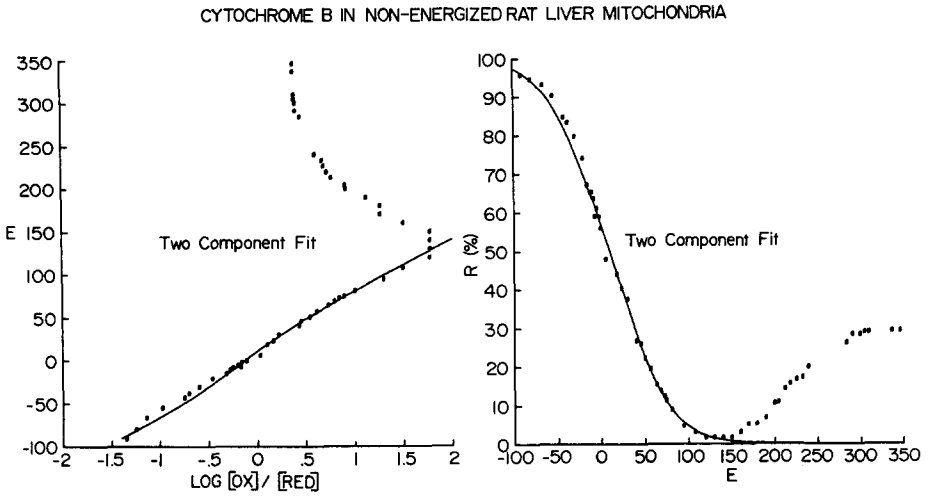

Fig. 8. The data in this experiment were collected in a repeat titration carried out 26 min after the addition of ATP in the experiment depicted in Figs 6 and 7. The data used for analysis were 38 pairs of voltage and corresponding absorbances in the range of +120 to −91 mV. An additional 22 pairs of voltage and absorbance data collected from +351 to +130 mV are shown in the figure but were not used for curve-fitting analysis because of their obvious anomalous character (i.e. absorbance decrease associated with decreasing voltage). Reduction was accomplished with trace amounts of ascorbate to about 0 mV and traces of NADH to −100 mV. The observed data are represented as open circles and the computed best fit for two components (a three-component fit could not be obtained) as solid lines. A plot of voltage, (E(mV)), vs. log (oxidized/reduced) is seen in the left panel and a plot of percent reduced R vs E in the right panel. The root mean square error for computed and observed "percent reduced" was 2.7 %.

With ATP-energized mitochondria a quantitatively small anomalous response was sometimes encountered in the voltage region +270 to +180 mV. When this happened, the computed midpoint potential for the high-potential component was raised to almost 400 mV whereas the middle and low potential components were hardly affected. A correction applied to the questionable data (based on data collected in experiments not showing the anomalous behavior) brought the computed voltage to the expected level. Of the seven experiments shown for the three component solution in Table III, only one had the anomalous behavior and correction applied. Several other experiments with anomalous behavior were encountered but not used for the summaries shown in the table. It is also important to emphasize that, in our hands, the high potential species had a rather transient existence. Immediately after collecting data from +350 mV to −100 mV in the presence of ATP, a repeat titration, failed to reveal any trace of the high potential component. In fact the data in Table III listed for MOPS buffer and non-energized mitochondria were mostly obtained in repeat titrations after the initial rapid titration following the addition of ATP. We estimate the total lifetime of this component to be less than 20 min after ATP addition. We measured the level of ATP present during the experiment and found that of the 6 mM ATP present at the time of addition, about 2.5 mM was present at 5 min and about 0.4 mM at 10 min. This high rate of ATP hydrolysis is compatible with a properly mediated system according to Wikström and Lambowitz [18]. The ATP is consumed in a continuous process of reversed electron flow between cytochromes $c+c_1$ and cytochrome *b*. Another potential problem was the fact that either in the presence or absence of 5 μM rotenone, the endogenous substrates, in the presence of ATP caused an extremely rapid reduction of cytochrome *b* and change of voltage. These changes occurred so fast that we wonder whether the state of reduction of the cytochrome always reflected an equilibrium with the measuring electrode.

There are certain very marked characteristics which identify and distinguish non-energized and energized forms of cytochrome *b**. In the non-energized case, only two components are readily fit by the computer. The midpoint potential of the more positive component is between 30 and 100 mV** and that of the more negative component is close to but usually less than 0 mV. In the voltage range +350 to +150 mV the anomalous behavior, discussed above, is seen. Non-energized cytochrome *b* has been encountered in phosphate buffer and under the following circumstances in MOPS buffer: (1) A repeat titration following a titration in the presence of ATP; (2) Using mitochondria aged by stirring 1 h in air at room temperature; (3) A third successive titration carried out in the absence of added ATP.

Energized mitochondria do not display the characteristics listed above for non-energized mitochondria. In addition, the data are readily fit both to two- and three-component solutions, but the closeness of fit is much better in the three- than the

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* The terms "energized" and "non-energized" are borrowed from Wilson et al. [1]. These designations are useful in referring to the two types of responses of cytochrome *b* to variations of voltage (i.e. potentiometric titrations). We do not necessarily agree with Wilson et al. that the cytochrome *b* is actually in a different energy state when either ATP or dinitrophenol is added.

** For the two different preparations of mitochondria used for experiments reported in Table III, the midpoint potential of the middle component was as shown. With a third preparation of mitochondria, the average of six experiments was 100±7.1 mV for the same component. This variation may reflect the state of association of this species of cytochrome *b* with the membrane.

two-component solution. The third component has a midpoint potential above +230 mV. Using these criteria, we have found that energized cytochrome *b* is present in the absence of added ATP even on two successive rapid titrations. If, indeed, the cytochrome is energized, it has to be obtaining its energy from electron transport. This would mean that electrons are pulsing through an energy transducing site of the electron transport chain more rapidly than through the short circuits to the platinum electrode provided by the soluble mediators. This situation would lead to errors in the determination of midpoint potentials. Whereas a titration from +350 mV to about 0 mV in the case of *E. coli* fractions took between 1 and 2 h; with rat liver mitochondria, spontaneous reduction from endogenous substrates usually traversed this voltage range in less than 5 min. The voltage range from +350 to +150 mV was covered in less than 1 min. The presence of $6.7 \cdot 10^{-4}$ M 2,4-dinitrophenol did not completely prevent the initial transient appearance of high potential cytochrome *b* in the presence or absence of ATP. Once again, during the initial very fast endogenous reduction phase, the speed of electron passage across the transducing site may have been able to generate a short-lived energized form of cytochrome *b* even in the presence of dinitrophenol. The effectiveness of the added dinitrophenol as an uncoupler was shown by the hydrolysis of 95 % of added 6 mM ATP in 6 min and the rest by 15 min. The quantity of short-lived high potential cytochrome *b* seen in the presence of dinitrophenol was 25–50 % of that seen in its absence.

Before we were aware of the transience of the high-potential species of the mitochondria we routinely stirred our samples in air at room temperature for 30–60 min to lower the level of endogenous substrates, then bubbled argon through the suspension for 20 min to insure anaerobiosis. ATP was added at various times from 10 min to 2 h after the start of the experiment. Our successful demonstrations of the high-potential species in mitochondria were accomplished on a much more rigorous schedule as follows: (1) Start of argon bubbling through cold suspension of mitochondria in reaction vessel. (2) Mediators (16 μM each) added at 2 min. (3) K_3FeCN_6 added at 7 min to bring the voltage to about +380 mV. (4) Second addition of mediators (16 μM each) at 11 min. (5) ATP (6 mM) added at 13 min.

Experiments were performed with *E. coli* under the same schedule. The results were no different from those obtained with the longer time schedule. In the data shown in Table II, two experiments for each of the 4 *E. coli* fractions shown were performed according to the above schedule. ATP levels were monitored in these experiments and found to be higher than those found during the experiments with mitochondria (i.e. about 3–4 mM at 10 min and about 2–3 mM at 30 min).

In the experiments with mitochondria reported in Table III, we used a higher concentration of mitochondria than Wilson and Dutton [6] and we did not have mannitol present as an osmotic stabilizer. We did not use EDTA partially chelated with Fe^{2+}, which they referred to as an optional redox buffer [6]. Although we were able to reproduce their observations, the short life of the high potential component, the extremely rapid endogenous rate of reduction, and the optical anomaly encountered in the non-energized condition, caused sufficient concern that we conducted another series of experiments with mitochondria under conditions resembling theirs. A particular consideration was that the presence of EDTA may have conferred longer life on the energized form of cytochrome *b* and perhaps have slowed the rapid rate of endogenous reduction by virtue of its ability to inhibit Mg^{2+}-stimulated

ATPase. We used a medium containing 0.22 M mannitol, 0.05 M sucrose, 50 mM MOPS, 5 μM rotenene, 0.6 mM EDTA and 6 mg/ml mitochondria. Although the lower concentration of mitochondria and presence of EDTA did reduce ATPase activity, the rate of ATP hydrolysis was still high (26 % hydrolysis in 5 min and 33 % in 10 min in the presence and 38 % hydrolysis in 5 min and 53 % in 10 min in the absence of EDTA). With or without EDTA, the rate of endogenous reduction was just as rapid as seen under the previous conditions. ATP was not able to induce the appearance of the high potential component, if it was added directly after an initial fast endogenous substrate-induced reduction in the absence or presence of EDTA. When ATP was added at the beginning of the experiment, the high-potential component was seen in the absence of EDTA but not in its presence. The optical anomaly in the voltage range of about +350 to about +150 mV was seen for non-energized mitochondria in the presence and absence of EDTA.

DISCUSSION

We have described the presence of three cytochromes b_1 in the electron-transport chain of *E. coli*. Neither the relative amounts nor midpoint potentials were changed in the presence of ATP or 2,4-dinitrophenol. The lowest-potential form (E_m approximately −50 mV) appeared to be completely extractable from the membrane by 1 % deoxycholate. This species may represent the cytochrome b_1 solubilized and crystallized by Deeb and Hager [19]. The middle-potential form (E_m approximately +110 mV) was extracted to the extent of about 50 % and the high-potential form (E_m approximately +220 mV) about 10 % by the use of 1 % deoxycholate. We have previously described the solubilization and fractionation of the electron-transport chain of *E. coli* and the isolation of three separate forms of cytochrome b_1 [20]. One appears to be separate from other members of the electron-transport chain; another is contained in a complex with succinate dehydrogenase and the other in a complex with *E. coli* cytochrome oxidase. We will try to determine if these three forms account for the three redox potentials derived from the current work.

In our studies with rat liver mitochondria, we have confirmed the apparent existence of two forms of cytochrome *b* in the non-energized system. In the energized system, the redox properties of cytochrome *b* are dramatically different. A two-component fit of the data shows the disappearance of the low-potential form (E_m approximately −9 to −30 mV) and the appearance of a new high-potential form (E_m approximately +240 to +280 mV). The other form (E_m approximately +30 to +70 mV) seems to remain unchanged. Wilson et al. and Chance et al. interpret these findings as a demonstration of the ATP-dependent transformation of an energy-transducing species of cytochrome *b* (b_t) from a low- to high-energy state. Similar observations have been reported for mitochondrial and submitochondrial preparations from beef and pigeon heart, although non-energized beef heart appears to have three cytochromes *b* of potentials about +135, +40, and −110 mV. Berden et al. [21] have reported that ATP affects the redox potentials of both the highest (they find an E_m of +154 mV) and the middle potential species (E_m +28 mV) of beef heart mitochondria. The lowest-potential species was not observed in their experiments. Allowing for the possibility that ATP affects two forms of cytochrome *b*, an alternative explanation of the data of Wilson et al. is that the −30-mV species of non-energized rat

liver mitochondria was converted to the +35-mV species by ATP and the +30-mV species to the +245-mV form. We find, however, that the data obtained with energized rat liver mitochondria are better fitted to a three component rather than a two component system. The results of this treatment suggest the possibility that the potential of neither of the original two cytochromes is changed upon energization but that a new high-potential species makes its appearance. We have also observed certain technical aspects of the experiments with mitochondria which add to the uncertainty of interpreting the results. For example, with non-energized mitochondria we have encountered an absorbance anomaly in the voltage region from +150 to +350 mV thus obscuring information on the possible presence of a +240 to +280 mV species (see Fig. 8). With energized mitochondria, the rates of cytochrome *b* reduction and voltage decrease caused by endogenous substrates were so rapid that a possible lag (i.e. non-equilibrium) between the two processes may exist.

In connection with our finding of three possible species of cytochrome *b* in energized rat liver mitochondria it is significant to note that Berden et al. [21] also resolved their titration curve for energized beef-heart mitochondria into three components. Their high-potential species (E_m +270 mV), and middle potential species (E_m +118 mV) correspond very well to the high (E_m +260 mV) and middle (E_m +96 mV) potential species we observe in rat liver mitochondria. However, the low-potential species in beef heart mitochondria was reported to be E_m +48 mV compared to our value of −20 mV for rat liver mitochondria. It should also be noted that Berden et al. [21] did not observe an optical anomaly in the high voltage region of the titration of non-energized beef-heart mitochondria and that they obtained the same total absorbance (*A* 562–575 nm) for energized and non-energized mitochondria. The apparent similarities and differences for the results obtained from these two species of mitochondria require further study.

In spite of the reservations we have discussed, we do not discount the interpretation of Chance and Wilson and their collaborators on the possible existence of an energy transducing cytochrome. One possibility for our inability to find an influence of ATP on the b_1 type cytochromes of *E. coli* could be that a very high phosphate potential is required to sustain the high-energy form and that the level of ATPase activity reduces its lifetime to less than the time of our experimental procedures. The individually isolated three species of cytochrome b_1 which we have described may have much lower levels of endogenous ATPase activity. We plan to look for ATP effects on these fractions.

A potentially significant finding of the current work was the energy-dependent optical anomaly in the voltage region of about +350 to +150 mV in the experiments with rat liver mitochondria. This could be due to interference introduced either by another chromaphore contributing to the absorbance at one or both of the wavelengths used for determining cytochrome *b* or to a physical change in the state of the membrane itself. The energy-dependent character of the phenomenon suggests that it could be important in energy conservation.

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