THE OXIDATION-REDUCTION STOICHIOMETRY AND
POTENTIAL OF MICROSONAL CYTOCHROME*

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In the preceding report (1) we have described the isolation, as an acidic protein of relatively low molecular weight, of the cytochrome component of rabbit liver microsomes. Of the various mammalian cytochromes, known from their spectra in crude preparations or intact cells, only cytochrome c has been previously isolated in a form which has permitted molecular characterization (2–4). Two properties of cytochrome c, its standard potential and its behavior as a univalent electron acceptor (5), have been major considerations in theories of metabolic electron transport. This paper deals with the potential and the stoichiometry of the oxidation and reduction of the microsomal cytochrome.

Methods

Potentials of the cytochrome were calculated from equilibrium measurements with oxidation-reduction couples of known standard potential. The cytochrome was prepared and analyzed by methods which have been described (1). Of the oxidation-reduction dyes in suitable potential range for equilibrium measurements, indigo tetrasulfonate, which has been well characterized potentiometrically (6), proved most satisfactory. The indigo sulfonates used were samples prepared and analyzed by Dr. P. W. Preisler. The second equilibrium system used was that established between the cytochrome and mixtures of ferrous and ferric oxalate. Potentials of the ferrous-ferric oxalate couple were reported by Michaelis and Friedheim (7). Ferrous and ferric oxalates have been used in cytochrome equilibria by Hill (8).

Anaerobic Cuvettes—Since many of the reaction systems studied were autoxidizable in air, anaerobic conditions had to be maintained. Two types of reaction vessel were employed. The first consisted of a square Corex cuvette of 1 cm. light path fused to a Thunberg bulb and side arm. The second type of cell was a 3 ml. Corex or silica cuvette with a ground glass joint and upper assembly as illustrated in Fig. 1. This type of cell was used in anaerobic titrations. Additions were made with a calibrated

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micro syringe and a No. 22 needle inserted and left in position in the rubber injection port. Increments of 0.02 ml. had a precision of about ±5 per cent, but, since straight lines could be fitted to a sequence of five to fifteen points, the over-all precision was better than 5 per cent.

In practice a small volume of cytochrome solution was pipetted into the cell. The upper assembly was inserted and the system was alternately evacuated on the water pump and flushed with nitrogen. Buffer, deaerated

with a vigorous stream of nitrogen, was added from a syringe through the injection port. The system was again alternately evacuated and flushed several times. Nitrogen was left in the gas space during an experiment. Even though quite small volumes of reagent were added in oxidative titrations, the solutions employed had to be deoxygenated. Mixing, after addition of reductant or oxidant, was effected by inversion of the cuvette assembly. With Linde nitrogen and the above precautions, the titration blanks were negligible, and the reduced cytochrome was stable.

Leuco Dyes—Dye solutions of somewhat higher than the desired final concentrations were reduced with hydrogen and a supported palladium
catalyst. These reductions were carried out with the solutions in the cylinder of a 5 or 10 ml. syringe through which a stream of hydrogen gas was bubbled. After reduction, the plunger was rapidly inserted and the hydrogen bubble and some of the solution were ejected through the filter assembly illustrated in Fig. 2. When not in use the needle was sealed by insertion in a soft rubber stopper. Concentrations of the total and reduced dye were determined spectrophotometrically before and after reoxidation. Final concentrations were adjusted by sucking the appropriate amount of deoxygenated buffer into the syringe. In equilibrium titrations an excess of the reduced dye was injected directly from the storage syringe into the anaerobic cuvette containing the cytochrome solution. A series of equilibria was then established by addition of increments of standard ferricyanide.

### Table I

**Millimolar Absorption Coefficients Used in Equilibrium Calculations**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>$E_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>590 mp</td>
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<tr>
<td>Indigo tetrasulfonate, oxidized</td>
<td>5.5</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>0</td>
</tr>
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<td></td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>0</td>
</tr>
<tr>
<td>Microsomal cytochrome, oxidized</td>
<td>6.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>170</td>
</tr>
</tbody>
</table>

When the leuco dye was used as a reductive titrating agent it was first transferred to a smaller syringe through the coupler shown in Fig. 2, B. Other solutions used in anaerobic work were deoxygenated and stored in small stoppered separatory funnels fitted at the tips with rubber injection ports for the removal of samples with a syringe and needle.

Absorption Coefficients—Absorption coefficients, determined on dye solutions standardized by weight, were checked by stoichiometric titration of the leuco dye in anaerobic cuvettes with standard ferricyanide. In a 2 electron change the titration slope, $\Delta \log(\text{I}_0/\text{I}) \, \mu\text{eq}^{-1} \, \text{ml}^{-1}$, is one-half the difference between the millimolar absorption coefficients of the oxidized and reduced forms of the dye at the wave-length investigated. The absorption coefficients of dye and cytochrome used in the equilibrium measurements are given in Table I. From optical density measurements at three wave-lengths, it was possible to calculate the concentrations of three of the four reacting components in the cytochrome-dye equilibrium. The correct ab-
solute values of the absorption coefficients are unimportant in the equilibrium measurements, provided that the ratios between them are accurately known.

EXPERIMENTAL

Oxidative and Reductive Titrations—Buffered cytochrome solutions in anaerobic cuvettes were reduced by addition of increments of deoxygenated solutions of sodium hydrosulfite, leucoanthraquinone-2,7-disulfonate, or leucoindigo disulfonate. At pH 5.5, reduction could be effected by injection of 0.02 ml. of potassium borohydride, 20 mg. per ml. at pH 9, into the deoxygenated cytochrome solution. At the acid pH, the borohydride rapidly decomposes, reducing the cytochrome and liberating the hydrogen. After reductions with borohydride, the cuvette was repeatedly evacuated and flushed with nitrogen until no more bubbles were formed. Reductions were also carried out enzymatically with reduced diphosphopyridine nucleotide and the microsomal enzyme described in the following report (9). Cytochrome reduced by these various methods between pH 5 and 8 was titrated with standard ferricyanide. In those cases in which an excess of reducing agent was employed, there was an initial plateau in which increments of ferricyanide had no effect upon the spectrum. When excess reducing agent was consumed or if none was initially present, the optical densities at wave-lengths affected by the state of oxidation changed linearly with increments of ferricyanide until the cytochrome was completely re-oxidized. The results of a titration after hydrosulfite reduction are shown in Fig. 3. In this titration the optical density of the solution was measured at 413, 423, 527, and 555 m\(\mu\) after each increment of ferricyanide. Within the limits of experimental error the curves, after correction for volume changes, are linear, show no significant breaks, and begin and end abruptly. The linearity of the titration and the fact that the absorption bands move in unison indicate the presence of a single heme protein. Moreover, no evidence has been obtained in such titrations for the existence of any intermediate spectra that cannot be accounted for as the simple sum of the contributions of the fully oxidized and fully reduced forms present.

Similar titrations have been carried out by starting with the cytochrome in the oxidized form and by using hydrosulfite or leucoanthraquinone-2,7-disulfonate as the titrating agent. Freshly prepared deoxygenated solutions of these substances were standardized immediately before and after use by spectrophotometric titration of indigo tetrasulfonate. Occasional reductive titrations of the cytochrome exhibited a variable initial plateau owing to the presence of traces of oxygen or other reducible impurities. No consistent evidence could be obtained by this method for the existence of any reducible group on the protein other than the prosthetic group. The
reductive titration curves were linear with the same slope exhibited in the oxidative titrations. Several consecutive cycles of reduction and oxidation were carried out with the same or different reagents with no significant change in the slopes.

The slope of an oxidative or reductive titration curve, \( \Delta \log (I_0/I) \, \text{mEq}^{-1} \, \text{ml}^{-1} \), for a cytochrome exhibiting a 1 electron change, should be numerically equal to the difference between the millimolar absorption coefficients of the oxidized and reduced forms. In twenty-five oxidative titrations in the pH range 5 to 8 on six different cytochrome preparations, the average slope at 423 m\( \mu \) was 91 with a standard deviation of 7. The slopes of a smaller number of reductive titrations fell within these limits. The difference in millimolar absorption coefficients at this wave-length is 105. To what extent the deviation between these figures results from inaccuracies in the methods or from interfering groups or impurities is not yet known. The oxidation and reduction correspond to the removal and addition of 1 electron, determined with an over-all accuracy of \( \pm 10 \) per cent.

![Diagram of oxidative titration of microsomal cytochrome](image)

**Fig. 3.** Oxidative titration of microsomal cytochrome with potassium ferricyanide after reductive titration with sodium hydrosulfite. The ordinate, \( D_\lambda \), is the optical density at the wave-length, \( \lambda (\text{m}\mu) \), which is indicated numerically at each curve. The solution contained 0.05 M potassium phosphate buffer, pH 7.0. The initial volume was 2.25 ml., and reduction was effected by stepwise addition of 0.03 ml. of sodium hydrosulfite solution under anaerobic conditions. Reoxidation was effected with 2 \( \times \) \( 10^{-4} \) M potassium ferricyanide. All optical density readings are corrected for dilution by the oxidant.
titration of reduced cytochrome c, followed at 550 m$\mu$, showed slopes of 18.4 to 18.6, whereas the difference in millimolar absorption coefficients of the reduced and oxidized forms at this wave-length has been reported to be about 18.9 (10).

Equilibrium with Indigo Tetrasulfonate—Indigo disulfonate had too negative a potential to establish easily measurable equilibria with the free cytochrome, but indigo tetrasulfonate, $E'_0 = -0.046$ at pH 7, established measurable equilibria between pH 5 and 7. A 10- to 20-fold excess of the reduced dye was injected into a buffered solution of the cytochrome in an anaerobic cuvette. Optical densities were read at 413, 423, and 590 m$\mu$. A syringe containing approximately $10^{-4}$ M ferricyanide was inserted in the injection port and readings at the above wave-lengths were taken after each successive increment of 0.02 ml. of ferricyanide. Equilibria were established and stable before the first reading could be taken. The concentration of the oxidized dye was computed from the reading at 590 m$\mu$. The concentration of the reduced dye at each equilibrium point was obtained by difference between this value and the total dye concentration checked at the end of the experiment from the 590 m$\mu$ reading after complete reoxidation. The optical density readings at 413 and 423 m$\mu$ were corrected for the dye contributions, and the corrected values were used to compute reduced and oxidized cytochrome concentrations by solution of the simultaneous equations

\begin{align*}
D_{413} &= C_o E_{413,o} + C_r E_{413,r} \\
D_{423} &= C_o E_{423,o} + C_r E_{423,r}
\end{align*}

where $D$ is the corrected optical density at the designated wave-length, $E$ is the millimolar absorption coefficient, $C$ is the concentration of cytochrome, and the subscripts $o$ and $r$ refer to oxidized and reduced forms. As a check on the measurements and calculations, the sums of the concentrations of the two forms of cytochrome must agree with the initial concentration and the final concentration rechecked at 413 m$\mu$ after complete reoxidation.

The results of the equilibrium titration at pH 5.5 are shown in Table II. $K'$, the equilibrium constant, is seen to hold within reasonably close limits between 25 and 83 per cent oxidation of the cytochrome when the reaction is formulated as

\begin{align*}
(\text{Dye})_o + 2(\text{cytochrome})_o & = (\text{dye})_r + 2(\text{cytochrome})_r \\
K' &= \frac{[\text{dye}]_r}{[\text{dye}]_o} \times \left( \frac{[\text{cytochrome}]_o}{[\text{cytochrome}]_r} \right)^2
\end{align*}

Thus the equilibria are in accord with the titration stoichiometry and indicate a 1 electron change in the cytochrome.
The equilibria may also be expressed in terms of potentials. In this method the dye is treated as an oxidation-reduction indicator. At each equilibrium point the potential of the indigo tetrasulfonate referred to the normal hydrogen electrode is given by the relation

\[ E = E_0' + \frac{RT}{2F} \ln \left( \frac{[\text{Dye}]_o}{[\text{Dye}]_r} \right) \]  

and can be computed since \( E_0' \) as a function of pH is known (6) and the concentration term is measured. At equilibrium, the cytochrome potential is the same as that of the dye. Accordingly in Fig. 4, the computed value of \( E \) at each equilibrium point is plotted against the fraction of the cytochrome in the oxidized form. The solid curves are the theoretical curves for a substance undergoing a 1 electron change, corresponding to a numeri-

<table>
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<th>Equilibrium No.</th>
<th>[Dye]_o</th>
<th>[Dye]_r</th>
<th>[Cytochrome]_o</th>
<th>[Cytochrome]_r</th>
<th>[Cytochrome]_o + [Cytochrome]_r</th>
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<tr>
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cal factor of 1 instead of 2 in the denominator of the second term on the right in Equation 5. As would be expected from the data in Table II, the points fall on the potential curves for a 1 electron change. $E'_o$ is the potential at the mid-point of the curve. As the pH is increased, the $E'_o$ of the cytochrome and that of the dye diverge so that the range of the titration curve that can be covered by equilibrium measurements becomes progressively more limited. At pH 6.9 it is necessary to extrapolate to the mid-point from experimental points which do not quite fit the theoretical curve.

*Equilibria with Ferrous and with Ferric Oxalate*—The $E'_o$ of the ferrous-ferric oxalate couple is close to zero below pH 7 and is independent of pH. Near pH 7, the $E'_o$ curve assumes a slope of $-0.06$ volt per pH unit according to potentiometric measurements (7). Using the equilibrium titration method described in the preceding section, we measured equilibria between ferrous and ferric oxalate and the indigo tetrasulfonate couple over the pH range 5.2 to 8.5. The values of $E'_o$ for the ferrous-ferric oxalate couple calculated from these measurements were in fair agreement with the potentiometric values. Corresponding experiments were therefore undertaken with the cytochrome in place of the dye.

*Fig. 4. Potential of microsomal cytochrome calculated from equilibria with reduced and oxidized indigo tetrasulfonate at 26°.* Temperature in these and other experiments was controlled with thermal spacers around the carriage compartment of the spectrophotometer. These were connected to a circulating water bath.
Buffered solutions of the cytochrome containing 0.5 M sodium oxalate and 0.001 M ferric ammonium sulfate were prepared in anaerobic cuvettes. Measured increments of freshly prepared deoxygenated ferrous ammonium sulfate were then added from a syringe, and readings were taken after each addition at 413 and 423 mμ. The potential at each equilibrium point was calculated from the \( E_0' \) and known concentrations of ferric and of ferrous oxalate by the relation analogous to Equation 5 for a 1 electron change. Reduced and oxidized cytochrome concentrations were computed as before by Equations 1 and 2. The \( E_0' \) values of the cytochrome derived from these results and from the indigo tetrasulfonate equilibria are shown in Fig. 5 as a function of pH. Between pH 5.2 and 6.3 the results by the two methods are in fair agreement and indicate a potential, \( E_0' \), between +0.03 and +0.025 volt for the free cytochrome. Since the criteria for equilibria were satisfactory and since the concentrations of all of the reacting components were measured, the assignment of the cytochrome potential depends chiefly upon the published standard potentials of the reference compounds. Internal consistency between the published values for ferrous-ferric oxalate and indigo tetrasulfonate was established by titrating them against each other. At pH values above 6.5 there is indication of a negative slope in the \( E_0' \) versus pH curve for the cytochrome which is

![Fig. 5](https://example.com/fig5.png)

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**Fig. 5.** The apparent standard potential, \( E_0' \), of the microsomal cytochrome as a function of pH at 26°C. ○, indigo tetrasulfonate equilibria; ●, ferric-ferrous oxalate equilibria. The upper curve is from the data of Rodkey and Ball (5).
smaller than that predicted for either a 1 or a 2 electron change. Because of limitations of the methods in this pH region, we are reluctant to accept or interpret any apparent $E'_{0}$ versus pH slope for the cytochrome at the present time. Spectral criteria and stoichiometric titrations are in accord with a 1 electron change for the cytochrome, both in the acid and in the alkaline pH regions.

DISCUSSION

The potential of the free microsomal cytochrome at pH 7 is more than 0.1 volt higher than values reported for the pigment in particulate suspension (11, 12). The question is naturally raised as to whether the cytochrome has become altered during its isolation, possibly in the incompletely defined enzymatic step in its preparation (1). It should be noted, however, that no evidence for such a change is provided by comparison of the spectra of the free and bound forms of the cytochrome (1). As shown in the following report (9), the cytochrome also has specific enzymatic properties. Aspects of similar problems concerned with the status of free cytochrome c are discussed in a recent paper by Keilin and Hartree (13).

A difference in the apparent standard potential of the free and bound forms of a reversibly oxidizable and reducible substance is not without precedent. An immediate case in point is the ferric-ferrous oxalate system employed in the present work. The ferric-ferrous couple in oxalate complex has a potential about 0.8 volt lower than values obtained in the absence of such a complex-forming agent. The large shift in the equilibrium of the alcohol dehydrogenase reaction in the presence of stoichiometric concentrations of the enzyme results from differential complex formation of the reduced and oxidized forms of diphosphopyridine nucleotide by the protein (14, 15). A shift would occur in the presence of any differential complex-forming agent, not necessarily the enzyme catalyzing the reaction. In a thermodynamic treatment, the equilibrium shift emerges as a change in standard potential of the nucleotide. The potential shift which occurs on liberation of the microsomal cytochrome from its particulate complex may perhaps be attributed to a differential interaction of the reduced and oxidized cytochrome with one or more components of the microsomal particle. This does not eliminate the problem of possible alteration of the protein during its isolation but raises the question of the nature of the binding. The potential shift may be of purely thermodynamic interest, or it may reflect fundamental differences in the mechanisms of reaction of the free and bound cytochrome.

The free microsomal cytochrome, like cytochrome c, behaves as a univalent electron donor and acceptor. The idea that electrons move, one at
a time, through a cytochrome sequence, extrapolated from the behavior of cytochrome c, has posed one of the many difficult problems in the understanding of the chemical coupling between electron transport and biochemical synthesis, since the latter process is most readily understood in terms of a 2 electron reaction.

SUMMARY

1. Methods are described for carrying out spectrophotometric, oxidative, and reductive titrations of autoxidizable compounds under anaerobic conditions. These methods have been applied to the low molecular weight cytochrome isolated from liver microsomes.

2. The absorption bands of the cytochrome move linearly and in unison with increments of an oxidizing or reducing agent. This is interpreted as evidence for the presence of a single heme protein.

3. Oxidation-reduction equilibria have been measured between the cytochrome and indigo tetrasulfonate and the cytochrome and ferrous-ferric oxalate. From the equilibrium measurements the standard potential, $E_0'$, of the cytochrome at pH 7 is calculated to be +0.02 volt.

4. From stoichiometric, oxidative, and reductive titrations and from oxidation-reduction equilibria the cytochrome behaves as a univalent electron donor and acceptor.

BIBLIOGRAPHY

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