## Experimental Section

**Materials.** All of the primary anilines were commercial samples except for 3-methyl-2,4,6-trinitroaniline, which was kindly provided by the late Dr. M. Jorgenson. These substances were recrystallized from ethanol or ethanol-water mixtures until they had constant melting points which agreed with accepted literature values.

2,4-Dimethoxyazobenzene was prepared by coupling diazotized aniline with *m*-methoxyphenol and then methylating the free hydroxyl group with dimethyl sulfate.[19] This coupling reaction produced two substances, both of which gave 2,4-dimethoxyazobenzene upon methylation; that fact plus their nmr spectra identified them as the positional isomers 2-hydroxy-4-methoxyazobenzene and 4-hydroxy-2-methoxyazobenzene. 2,4-Dimethoxyazobenzene was purified by alternate recrystallization from 95% ethanol and from hexane until its melting point was constant and in agreement with the literature value.

This preparation gave a mixture of *cis*- and *trans*-2,4-dimethoxyazobenzene, but isomerization of the cis isomer to the more stable trans form is acid catalyzed and very rapid at the acidities employed for the indicator measurements.[20] Thus, these measurements refer to *trans*-2,4-dimethoxyazobenzene only.

Deionized water was purified further by distillation from alkaline permanganate in glass apparatus. All other materials were best available commercial grades and were used without further purification.

**Density Measurements.** Solutions were prepared by pipetting 10-ml quantities of 95% ethanol into 50-ml volumetric flasks and then filling the flasks to the mark with aqueous sulfuric acid of the appropriate concentration. With concentrated acids, considerable heat was evolved during this dilution; the acid was therefore added in small portions and the flask was cooled between additions. In all cases, final volume adjustments were made with the flask and its contents in temperature equilibrium with a bath operating at 25.0 ± 0.05°.

Densities were determined using Weld pycnometers of 10-ml nominal volume; these were filled in the recommended way[21] while suspended in the 25° constant temperature bath. Weighings were performed on a Mettler type B6 semimicrobalance and were corrected for the effect of air buoyancy.[21] Each measurement was made in duplicate with each of two pycnometers; the results are therefore averages of four separate determinations.[10] Some density measurements of wholly aqueous sulfuric acid were also made; these agreed with published[22] values to within 0.001 g/ml.

A few density measurements of 20% ethanolic sulfuric acid have been made before,[3b] but the values reported are consistently lower than the present results by *ca.* 2%; the reason for this difference is not known.

**Indicator Measurements.** Stock solutions of indicators in 95% ethanol were prepared at concentrations (*ca.* $10^{-3}$ *M*) selected to give maximum absorbance readings. Aliquots of these solutions were then diluted with sulfuric acid as described above for density measurements, and spectra were recorded from 500 to 350 nm using spectrometers (Beckman DK-2 or Cary 11) with cell compartments thermostated at 25.0 ± 0.1°. Absorbances were estimated to 0.001 from the recorded traces at absorption maxima and also at positions 5 nm to either side. The values of $A$ so obtained were transformed into indicator ratios using the relationship $I = (A_B - A)/(A - A_{BH^+})$, where $A_B$ and $A_{BH^+}$ are the absorbances of solutions containing indicator completely in its basic and acidic forms, respectively. These limiting absorbances were measured at acidities at least 3 $H$ units to either side of the indicator $pK_a$. The spectrum of each solution was usually recorded three times, and most values of $I$ are therefore averages of nine measurements.[10]

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# Electrochemical Studies of Heme Proteins. Coulometric, Polarographic, and Combined Spectroelectrochemical Methods for Reduction of the Heme Prosthetic Group in Cytochrome *c*

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**Abstract:** The detailed electrochemical behavior of native horse-heart cytochrome *c* is described. This heme protein is shown to reduce at a variety of electrode materials producing freely diffusing ferrocytochrome *c* that is fully active in the cytochrome oxidase enzyme system. Adsorption of the protein onto the electrode surface has significant influence on the observed electrochemistry, but it does not cause electrode fouling or loss of the electrode's ability to transfer electrons. On the basis of these results, it is not possible to distinguish between an electron transfer mechanism involving charge conduction through the protein fabric and a mechanism wherein electron transfer occurs only at the exposed heme edge. The relaxation techniques developed here appear suitable for electrochemical study of high molecular weight proteins in general.

Because cytochrome *c*, a heme protein distributed widely in living organisms, has a central role in the electron transfer reactions of aerobic metabolism, the mechanism of reduction and oxidation of the protein iron is of great interest. Data from heterogeneous electrochemical experiments may yield information on redox stoichiometry, on equilibrium, on the transport of electroactive species to and from the electrode surface, and on the chemical reactions occurring between the electroactive species and other components in the solution phase. These data are obtained without alterations in the atomic composition of the solutions under study, since only electrons are added or removed. For these reasons we have begun what we believe is the first systematic application of the various methods of electrochemistry to an elucidation of the behavior of native cytochrome *c*. In addition these techniques can serve as clean, synthetic methods for the production of reduced or oxidized material without the required addition of other redox reagents. Electrochemical procedures may also be combined with spectrophotometric methods to increase the amount of

information which may be obtained, and to measure phenomena occurring at very fast times.[1-4]

Previous potentiometric studies of cytochrome $c$[5-9] utilizing mediators have provided internally consistent results for the free energy of electron addition to the ferriheme prosthetic group as a function of pH. The equilibrium potential of the cytochrome $c$ system has also been obtained spectrophotometrically using redox indicators in place of the heterogeneous electrode.[10,11] The two methods have given comparable results. The mechanism of the redox reaction has also been investigated spectrophotometrically.[12-14]

Early polarographic studies of solutions containing cytochrome $c$ were degradative in purpose.[15] The electrochemistry described did not pertain to reduction of the prosthetic group. The catalytic wave observed was shown[16-19] to be a "protein double wave" characteristic of cobalt reduction in the presence of proteins containing cysteine groups. Direct polarographic reduction of cytochrome $c$ was reported by Griggio and Pinamonti.[9] Three irreversible waves were seen, the first of which ($E_{1/2} \simeq -0.3$ V *vs.* sce) was attributed to reduction of the heme prosthetic group. The first successful attempt at macroelectrolysis of ferricytochrome $c$ to its ferro form was reported by Kono and Nakamura,[20] using a platinum electrode. The reduction process was masked by hydrogen evolution, however, and the ferrocytochrome $c$ produced was stated to be only 60% active with cytochrome $a_3$.

We describe here the results of a series of experiments designed to test the feasibility of using electrochemical relaxation methods for initiating and monitoring electron transfer reactions in native protein systems. The reduction of ferricytochrome $c$ at both mercury and platinum electrodes was studied, and spectrophotometric examination provided information on the nature and purity of the reduction products.

## Experimental Section

Materials. Preparations of horse-heart cytochrome $c$ were obtained through the courtesy of Dr. Grant Barlow, Abbott Laboratories, Chicago, Ill. All preparations were stored in a desiccator at 12° until used, and no further purification was attempted. Beef-heart mitochondria were obtained from Professors John Merola and Gerald Brierley of The Ohio State University.

Tris(hydroxymethyl)aminomethane (Tris) and glycine were obtained from Sigma Chemical Co. Cacodylic acid was obtained from Fisher Scientific Co. All other chemicals were Baker, reagent grade, and were used as purchased. Solutions were prepared with demineralized doubly distilled water.

Unless noted in the text, all experiments were performed in a pH 6.05 buffer of 0.05 $M$ Tris, 0.05 $M$ cacodylic acid, and 0.10 $M$ perchlorate ion. All solvents were 0.10 $M$ in buffer and 0.10 $M$ in perchloric acid and were adjusted to the stated pH with NaOH. Vacuum triple distilled mercury was obtained from Bethlehem Appatatus Co. (Pittsburgh, Pa.). Oxygen was spurged from all solutions with Burdett (Cleveland) high purity nitrogen presaturated with water.

Potential-current measurements were made with operational amplifier circuitry assembled on a G. A. Philbrick Model RP manifold (Dedham, Mass.), together with a Hewlett Packard Model 7001 AM X-Y recorder. Electrochemical experiments were performed at ambient laboratory temperature (22 ± 1°), unless otherwise specified. A continuous flow of nitrogen was maintained over the solution during electrolysis to prevent oxygen contamination. Background polarograms on deaerated solutions indicated no oxygen within the limit of detection (*ca.* $2 \times 10^{-6}$ $M$).

Coulometric studies were performed in a thermostated cell (25.2 ± 0.1°) consisting of a mercury pool working electrode, a dip-type saturated calomel reference electrode, and a second calomel electrode used as a current carrying auxiliary electrode. A Wenking Model "TR" Potenticstat (Brinkmann Instruments, Westbury, N. Y.) was used for potential control.

**Procedure.** Lyophylized cytochrome $c$ was weighed into a dry 25.0-ml volumetric flask, and the appropriate buffer added to dissolve the sample. The solution was then transferred to the polarographic cell and deaerated for 2-3 hr (a slow stream of nitrogen minimized foaming). Polarograms run on the background electrolyte solution containing no protein indicated complete deoxygenation within 1 hr at the same $N_2$ flow rate. All potentials are reported *vs.* the saturated calomel electrode.

For coulometry, buffer solution (25.0 ml) was added to the cell, deaerated with nitrogen for 1 hr, and preelectrolyzed at −0.3 V *vs.* sce for 12-15 hr, under a nitrogen atmosphere. A weighed sample of lyophylized cytochrome $c$ was then added to the cell, and deaeration continued for 10-15 min before reapplying the voltage. The protein solution was electrolyzed at −0.3 V. During the course of the electrolysis aliquots were removed and spectra immediately recorded with a Cary 14 spectrophotometer. After 80-90% reduction, the cell was electrically disconnected and the protein reduced with sodium dithionite.

Potentiostatic studies were performed with the same apparatus used for coulometry. Solid sample was introduced after preelectrolysis as described above. A potential of approximately +0.10 V *vs.* sce was applied to a platinum gauze working electrode. When current ceased to flow (indicating apparent equilibrium) an aliquot was removed, and the visible spectrum immediately recorded. The ratio of reduced to oxidized protein was calculated using extinction coefficients for the 520- and 540-nm bands reported by Margoliash and Frohwirt.[21] The potential was stepped to a slightly more negative value, and the ratio of reduced to oxidized material again determined. This procedure was repeated until a voltage of −0.03 V was reached. At this point the voltage was steppted toward more positive values to verify the reversibility of the protein reduction. Comparable experiments were performed with a mercury pool electrode.

The enzymatic oxidation of reduced cytochrome $c$ was tested with beef-heart mitochondria disrupted by freezing and thawing, followed by sonic oscillation. Cytochrome $c$ was reduced with a mercury electrode at −0.03 V, with a platinum electrode at −0.02 V, or with sodium dithionite. In the case of chemical reduction the reagent was added and removed by dialysis in the absence of oxygen. The reduced protein was diluted into air-saturated buffer, and the enzymatic reaction begun by addition of the mitochondrial preparation. The oxidation of cytochrome $c$ at room temperature was monitored at 550 nm with a Pye Unicam SP 1800 spectrophotometer (Cambridge, England). Apparent first-order rate constants were obtained from semilogarithmic plots of the entire course of the reaction.[22]


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**Table I.** Correlation of Coulometric and Spectrophotometric Results[a]

| $t$ ($\times 10^3$ sec) | $Q$, C | $A_{550}$ | $A_{520}$ | $Q/FN$, equiv/mol | Fract red[b] | $n$[c] |
|---|---|---|---|---|---|---|
| 0 | 0.00 | 0.386 | 0.392 | 0 | 0 | |
| 7.35 | $2.77 \times 10^{-2}$ | 0.680 | 0.473 | 0.37 | 0.44 | 0.83 |
| 14.7 | $4.45 \times 10^{-2}$ | 0.867 | 0.529 | 0.61 | 0.73 | 0.83 |
| 22.0 | $5.38 \times 10^{-2}$ | 0.936 | 0.551 | 0.79 | 0.84 | 0.94 |
| | | 1.040[d] | 0.604[d] | | 1.00 | |

[a] The experimental procedure is described in the text; the protein concentration was 35.9 $\mu M$ (molecular weight of cytochrome *c*, 12,363 g/mol). [b] Calculated as the fraction of the total absorbance change at 550 and 520 nm. [c] Calculated as $Q/FN$ divided by the fraction of reduced cytochrome *c*. [d] Protein reduced completely with dithionite.

Spectroelectrochemical measurements were made by adding deaerated cytochrome *c* solutions to a gold gauze thin-layer electro-optical cell similar to that described by Murray, *et al.*[23] The spectral cell was made from a Beckman Model UV-10 flow cell in which the gold gauze (750 line/in. of gold mesh, Beckbee Mears Co., St. Paul, Minn.) was placed between two 0.05-mm Teflon spacers. Electrical contact was made directly to the gold gauze working electrode and to a saturated calomel electrode which made solution contact through a flow port; the other flow port was sealed with a rubber stopper. A Cary 14 spectrophotometer was used to monitor spectral changes in the cell, while an operational amplifier-based polarograph (see above) was used to control potential and monitor current. The cell was placed into the spectrophotometer, which had suitable attachments for electrode leads without admitting light, and the studies commenced.

## Results

**Controlled Potential Electrolysis.** Since little is known of the voltammetry of any material as large as cytochrome *c*, it was essential to investigate carefully the most fundamental characteristics of the electron transfer process of cytochrome *c* at electrode surfaces. Controlled potential electrolysis at a mercury pool electrode was used to verify the products of the electrochemical reduction. A potential of −0.3 V *vs.* sce (negative of the cytochrome *c* standard potential) was applied to a three-electrode coulometric cell (see Experimental Section). At the outset of the electrolysis experiment, the cell contained a 36 $\mu M$ solution of 95% oxidized protein, as indicated by its optical spectrum (see Figure 1, top line Table I). Samples were removed for spectrophotometric analysis at convenient times during electrolysis, and the correlation between the spectrum of the cell solution and $Q$, the total coulombs of charge passed in the electrolysis, was determined.

The reduction of the protein required more time than reduction of a simple inorganic ion at comparable concentrations. In one experiment, 30% of the protein was reduced in 2 hr, whereas, with the same cell arrangement, only 1 hr was necessary for 90% electrolysis of Cu(II). This slow reduction may be due to adsorption of a layer (or layers) of protein onto the electrode surface inhibiting the mass transport of unreduced material. Additional effects of the surface activity of cytochrome *c* are apparent throughout these electrochemical studies and are described further on.

The observed spectral changes (Figure 1) suggest that ferrocytochrome *c* is formed during the controlled potential electrolysis. The observation of four isosbestic points indicates that only two absorbing species are interconverted and the sum of the concentrations of the two forms is not changed during the course of the electrolysis.[21] Comparison of $Q$ to the fraction of the cytochrome *c* reduced (see Table I) yields an estimate of $n$, the number of electrons transferred per mole of protein reduced. The value of $n$ obtained, though less than one. indicates that the reduction of cytochrome *c* is a one-electron process, which is consistent with previous chemical and potentiometric data.[7,10] This low estimate of $n$ was found consistently. Two systematic errors may contribute to these results: irreversible adsorption of some of the cytochrome *c* on the cell and electrode surfaces prior to start of electrolysis, and existence of a fraction of the protein that was not reducible under these conditions. Errors in our correction for the background current may be responsible for the slight apparent increase in $n$ at longer electrolysis times. Because the coulometric method yields a direct measurement of charge transferred, it is an absolute method, and these results may be considered a proof of previous conclusions based on potentiometric results.

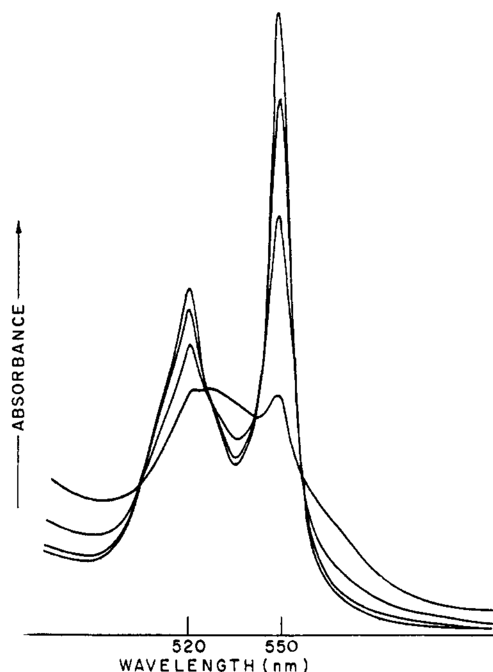


Figure 1. Visible spectrum of 36 $\mu M$ cytochrome *c* solution at successive stages of controlled potential reduction at −0.3 V for (1) 0, (2) 123, (3) 246, (4) 369 min.

The coulometric experiments suggest that it should be possible to carry out potentiostatic studies of the cytochrome *c* redox system in the absence of added mediators (such as the indigo sulfonates used previously[11]) and without need for the addition of potentially contaminating reducing agents such as dithionite.

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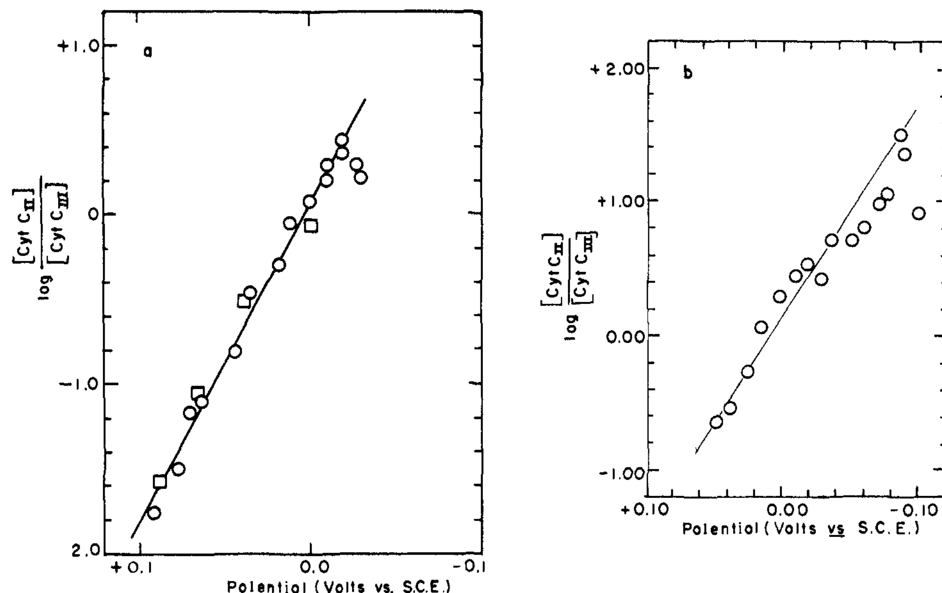


Figure 2. Equilibrium ratio of reduced to oxidized cytochrome *c* measured spectrophotometrically at various controlled potentials at (a) a platinum electrode and (b) a mercury-pool electrode.

Controlled potential electrolysis with no added mediators was used to generate an equilibrium ratio of oxidized and reduced protein, and the cell contents were analyzed spectrophotometrically to establish this ratio at each different potential applied. Potentiostatic data obtained using platinum gauze and mercury pool working electrodes are presented in Figure 2. Between one and several hours were required for the concentration ratios to reach equilibrium.

The cytochrome *c* system behaves in an essentially nernstian manner on platinum to a potential of about −0.02 V *vs.* sce (Figure 2a). At more negative controlled potentials the background current rises, and the ferri–ferro ratio can no longer be changed. This effect was not observed with a mercury electrode, suggesting a spurious reduction of some nonprotein component in the electrolyte solution which is kinetically favored on platinum under our experimental conditions. This spurious electrode process had no effect on the reproducibility or stability of the data at potentials positive of −0.02 V, since subsequently applying more positive potentials to the Pt electrode upon reaching the cathodic limit of −0.02 V yields the same nernstian behavior (Figure 2a, squares). Least-squares analysis of the data in Figure 2a gives values of −0.054 ± 0.002 for $RT/nF$, the reciprocal slope, and +0.003 ± 0.005 V for the intercept of the best straight line. A value of $RT/nF = -0.059$ is expected for a nernstian one-electron transfer. These compare favorably with the values −0.059 and +0.012 V reported previously from potentiometric work using electron transfer mediators and dithionite reduced enzyme.[22] Similar results were obtained with a mercury pool electrode (Figure 2b). Least-squares reciprocal slope and potential intercept of the nerstian plot were −0.062 and +0.010 ± 0.009 V, respectively. It is notable that with mercury the useful data extend over a more than 100-fold range in the ferri–ferro ratio.

The agreement between these potentiostatic results obtained without any electron transfer mediator and previous potentiometric and spectrophotometric experiments[5-9] is excellent, suggesting that protein adsorption does not affect the measurement of the formal potential. This demonstration of the efficacy of electrochemical modification of the prosthetic group in ferricytochrome *c* opens the way to the possible application of many other well-developed electrochemical methods. For example, we discuss below the application of polarographic and spectroelectrochemical techniques to obtain additional understanding of the cytochrome *c* redox system.

**Electrochemical Synthesis.** Use of electrochemistry as a synthetic method has received little attention in biochemistry. Its range of application as a synthetic tool is of essence limited to electron transfer reactions, but within that restricted class, electrochemical techniques offer an inherently clean and efficient route. To determine the usefulness of controlled potential electrolysis as a source of ferrocytochrome *c*, protein reduced at the mercury pool or the platinum gauze electrode was compared with dithionite produced material as substrates for the cytochrome oxidase activity of disrupted beef-heart mitochondria. At a fixed weight per cent of mitochondrial suspension, where the apparent rate constant for the oxidation of cytochrome *c* was independent of the catalyst concentration as expected, the apparent first-order rate constant obtained with the material reduced on either mercury or platinum was 120 ± 10% of the value obtained with the dithionite reduced protein (see Figure 3). We conclude from these results that the electrochemically produced material is as good as or better than the dithionite reduced material as a substrate of cytochrome oxidase.

**Polarography.** Polarography yields information on the kinetics and mechanism of the heterogeneous electron transfer proper, as well as on chemical processes undergone by the reactants and the products of the electrode reduction. We, therefore, investigated the reduction of cytochrome *c* at the dropping mercury electrode. It is a remarkable fact, of and by itself, that a well-formed polarographic wave can be observed

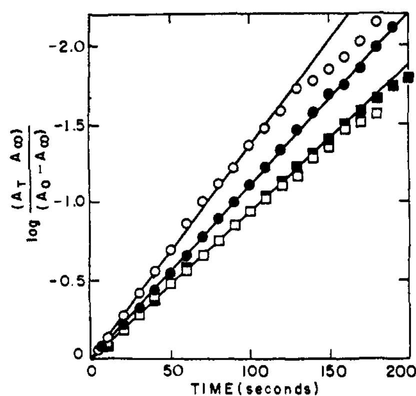


Figure 3. Kinetics of reaction between cytochrome oxidase and ferrocytochrome *c*: ●—●, 9.6 $\mu M$, cytochrome *c* reduced at Pt electrode; ○—○, 4.8 $\mu M$, cytochrome *c* reduced at Hg electrode; ◆—◆, 6.4 $\mu M$, cytochrome *c* reduced with dithionite; ◇—◇, 3.2 $\mu M$, cytochrome *c* reduced with dithionite.

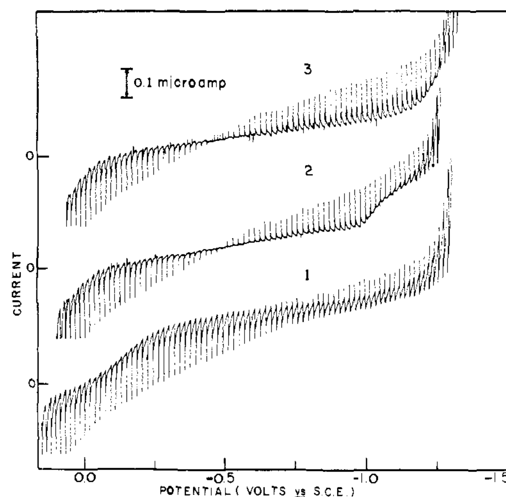


Figure 4. Polarogram of 64 $\mu M$ cytochrome *c* in Tris–cacodylate buffer, pH 6.05: (1) ferricytochrome *c*; (2) protein reduced at −0.3 V; (3) protein reduced at −1.2 V.

in solutions of cytochrome *c* (Figure 4). Substances of high molecular weight frequently are observed to adsorb tenaciously at the metal–solution interface with the result that electron transfer between electrode and electroactive molecules diffusing into the interface from the bulk solution is strongly inhibited, and waves are not readily found.[24] Protein adsorption is clearly indicated in our data by the irregular shape of the *i–t* curves (Figure 4), and by other observations to be presented shortly. The polarographic wave is due to the reduction of the heme iron of cytochrome *c*. This conclusion is based on the coulometric and potentiostatic results presented above, in which a one-electron reduction of the heme prosthetic group is strongly indicated.

The limiting current of the cytochrome *c* reduction wave is directly proportional to the bulk protein concentration above 10 $\mu M$ (Figure 5) at each pH investigated. Application of the modified Ilkovic equation[25] to the data in Figure 5 yields a value for the apparent diffusion coefficient of $0.5 \times 10^{-6}$ cm$^2$ sec$^{-1}$ for ferricytochrome *c* at pH 6.05 in Tris–cacodylate buffer. This estimate is significantly lower than the value of $0.95 \times 10^{-6}$ cm$^2$ sec$^{-1}$ determined by hydrodynamic techniques.[26] A layer of adsorbed material on the electrode surface could lower the apparent diffusion coefficient by decreasing the rate of mass transport of the electroactive species to the partially blocked surface.[24] Nonetheless, the overall electrode reaction must result in the conversion of freely diffusing ferricytochrome *c* to freely diffusing ferrocytochrome *c*, *i.e.*, reduction is not limited to material which is strongly adsorbed. Certainly the amount of charge passed during the lifetime of a single drop greatly exceeds that which would be required for reduction of a monolayer of protein on the electrode surface at the relatively high protein concentrations shown in Figure 5. If the cathodic current observed were due primarily to the production of surface immobilized material, then accumulation of the reduction product should interfere with mass transport causing a pronounced curvature in the current *vs.*

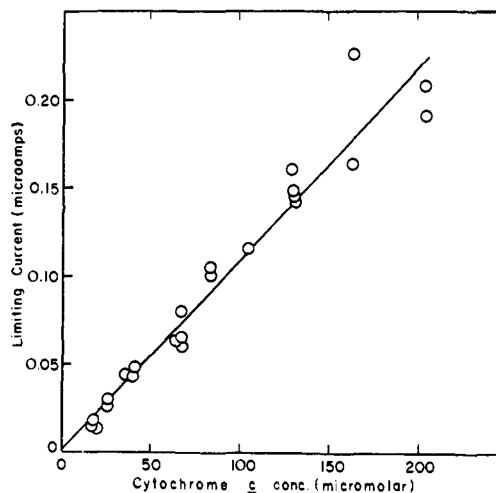


Figure 5. Dependence of diffusion current on concentration of cytochrome *c*, pH 6.05.

concentration plot, a result which is not observed. Further evidence for the diffusion-controlled nature of the polarographic wave is provided by plots of the logarithm of the limiting current *vs.* the logarithm of the mercury column height. The slope of such plots for solutions reported in Figure 5 was 0.53 ± 0.07.

The most likely alternatives to diffusion control of the limiting current in this case are: (a) mass transport controlled by the rate of a chemical reaction preceding electron transfer; (b) limitation of reduction to the protein adsorbed on the electrode surface. The log–log plot slope in case a should be zero and in case b should be one. We take the excellent correspondence between the slope observed and the value of 0.5, predicted for a diffusion-controlled process, to confirm our conclusions made on the basis of current dependence on protein concentration.

Nonetheless, protein adsorption effects are observed. First, as previously mentioned, the *i–t* curves are irregularly shaped; second, the waves are nonnernstian at pH values tested (Table II); and third, the value of $E_{1/2}$ is dependent on the protein concentration (Table III). The dependence of $E_{1/2}$ on protein concentra-

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**Table II.** Polarography of 100 $\mu M$ Cytochrome *c* at Varying pH Values

| pH | $E_{1/2}$, V *vs.* sce | Slope[a] | $i_d/C$, $\mu$A/m$M$ | Buffer |
|---|---|---|---|---|
| 2.50 | −0.40 | 0.16 | 0.5 | Citrate |
| | −1.13 | | 4.3 | Citrate |
| 4.00 | −0.34 | 0.18 | 1.2 | Citrate |
| 4.00 | −0.34 | 0.18 | 1.0 | Acetate |
| 5.00 | −0.22 | 0.15 | 1.5 | Acetate |
| 6.00 | −0.12 | 0.12 | 1.5 | Acetate |
| 6.05 | −0.13 | 0.12 | 1.6 | Tris-cacodylate |
| 7.08 | −0.21 | 0.16 | 1.4 | Tris-cacodylate |
| 8.00 | −0.21 | 0.10 | 0.7 | Tris-cacodylate |
| | −0.42 | 0.10 | 0.6 | Tris-cacodylate |
| 8.00 | −0.2 | | 0.6 | Borate |
| | −0.4 | | 0.6 | Borate |
| 9.00 | −0.41 | 0.8 | 0.9 | Borate |

[a] Reciprocal slope of plot of log $[i/i_d - i)]$ *vs.* $E$.

where $E_{1/2}$ and $E_{1/2}^0$ are the experimental and reversible half-wave potentials, respectively, and $R$, $T$, $\alpha$, $n$, and $F$ have their customary meaning. The lifetime of the drop and the time required for a predetermined surface coverage are $t_1$ and $\theta_1$, respectively. Finally, $a$ is an adjustable constant. The parameter $\theta$ is also expressed as

$$\theta = 1.85 \times \frac{10^6 \Gamma_I^2}{C^2 D} \quad (2)$$

where $\Gamma_I$ is the maximum number of moles per centimeters squared of the surfactant adsorbed to the electrode necessary to result in detectable inhibition of the electron transfer process, $C$ is the bulk concentration of this material, and $D$ is the diffusion coefficient.

**Table III.** Concentration Dependence of Polarographic Behavior

| Protein concn, $\mu M$ | $E_{1/2}$, V: 1st wave | $E_{1/2}$, V: 2nd wave | $E_{3/4} - E_{1/4}$[a]: 1st wave | $E_{3/4} - E_{1/4}$[a]: 2nd wave | $i_d$/concn, $\mu$A/m$M$: 1st wave | $i_d$/concn, $\mu$A/m$M$: 2nd wave |
|---|---|---|---|---|---|---|
| | | | pH 2.50 Citrate | | | |
| 106 | −0.400 | −1.130 | −0.12 | −0.06 | 0.47 | 4.25 |
| 132 | −0.475 | −1.130 | −0.15 | −0.05 | 0.45 | 2.68 |
| 165 | −0.530 | −1.130 | −0.17 | −0.06 | 0.69 | 2.29 |
| 207 | −0.550 | −1.130 | −0.13 | −0.06 | 0.37 | 1.67 |
| 258 | −0.580 | −1.130 | −0.16 | −0.05 | 0.40 | 1.24 |
| 323 | −0.595 | −1.130 | −0.15 | −0.05 | 0.32 | 0.94 |
| | | | pH 6.05 Tris-Cacodylate | | | |
| 17.7 | +0.005 | | | | 0.85 | |
| 20.3 | +0.005 | | | | 0.69 | |
| 26.2 | −0.005 | | −0.08 | | 1.07 | |
| 35.5 | −0.005 | | −0.09 | | 1.24 | |
| 40.1 | −0.009 | | −0.09 | | 1.07 | |
| 41.0 | −0.005 | | −0.11 | | 1.11 | |
| 63.7 | −0.065 | | −0.09 | | 0.99 | |
| 67.0 | −0.030 | | −0.09 | | 1.02 | |
| 83.7 | −0.070 | | −0.10 | | 1.22 | |
| 104.7 | −0.095 | | −0.10 | | 1.10 | |
| 129.4 | −0.100 | | −0.11 | | 1.19 | |
| 130.9 | −0.130 | | −0.09 | | 1.10 | |
| 163.6 | −0.155 | | −0.11 | | 1.19 | |
| 204.5 | −0.160 | | −0.12 | | 0.97 | |

[a] Plots of log $[i/(i_d - i)]$ were rectilinear as indicated in Table II.

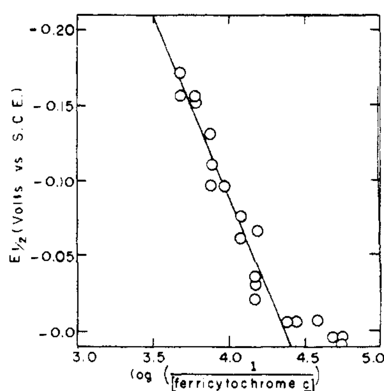


Figure 6. Dependence of half-wave potential of the cytochrome *c* reduction wave on log (cytochrome *c*), pH 6.05.

tion may be explained on the basis of an adsorbed protein layer retarding the rate of electron transfer. The shift of the half-wave potential due to adsorbed material is given by[24]

$$E_{1/2} - E_{1/2}^0 = 2.303RT/(\alpha nF) \log [a(\theta/t_1)^{1/2}] \quad (1)$$

Combining eq 1 and 2 yields

$$E_{1/2} = \beta + 2.303\frac{RT}{\alpha nF} \log \frac{1}{C} \quad (3)$$

with

$$\beta = 2.303\frac{RT}{\alpha nF} \log \left[a\sqrt{\frac{1.85 \times 10^6}{Dt}}\right] - E_{1/2}^0 \quad (4)$$

Plotting the observed value of $E_{1/2}$ *vs.* the negative logarithm of the protein concentration shows the expected linear relationship above a protein concentration of approximately 30 $\mu M$ (Figure 6). The observed correlation between experimental results and eq 3 supports our interpretation that cytochrome *c* adsorbed to the electrode surface induces nonnernstian behavior. The particularly low diffusion coefficient as calculated by the modified Ilkovic equation (see above) is also consistent with this interpretation.

Equation 1 fits the data in Figure 6 down to a concentration of approximately 30 $\mu M$ protein where the half-wave potential becomes nearly independent of cytochrome *c* concentration. Below this concentra-

tion, there is apparently no significant electron transfer inhibition. $\Gamma_I$ may then be estimated from eq 2 to be $4 \times 10^{-11}$ mol cm$^{-2}$ if $\theta = 4$ sec (the drop time) at 30 $\mu M$ protein concentration ($D = 1 \times 10^{-6}$ cm$^2$ sec$^{-1}$). Because $\Gamma_{max}$, monolayer coverage of the electrode surface, is calculated on the basis of the molecular dimensions to be roughly $7 \times 10^{-12}$ mol cm$^{-2}$ (assuming that the protein molecules pack with an approximate surface area of 60 Å × 40 Å or $2.4 \times 10^3$ Å$^2$), inhibition of the electron transfer reaction must begin after considerably more protein has diffused to the electrode surface than is necessary to provide monolayer coverage.

At pH 8 and above splitting of the polarographic reduction wave into two waves becomes evident. Although we have no explanation for this phenomenon, several authors[14,27,28] have suggested that cytochrome *c* exists in two thermodynamic states at alkaline pH. The presence of two protein species could be the basis of the polarographic behavior. In the low pH range an additional wave was observed at negative potentials of −1.0 V *vs.* sce. The limiting currents observed for this second wave were much greater than for the heme reduction wave occurring at more positive potentials. This fact and the very negative potential suggested that multielectron addition to some amino acid moiety[29] in the peptide chain might be occurring at low pH values. When the protein was subjected to a controlled potential electrolysis at −1.0 V, large structural changes were indicated by profound changes of the protein's absorption spectrum, although the heme bands may still be seen.

We have also looked for a polarographic oxidation wave using electrochemically or chemically synthesized ferrocytochrome *c*. No such wave was observed, the only polarographic activity being a reduction in the region of −1.0 V (Figure 4). The highly nonnernstian character of the polarographic reduction as reflected in the values of $E_{3/4} - E_{1/4}$ (Table III) or the slope of the current *vs.* potential plots (Table II) indicates the apparent irreversibility of the redox reaction within the time scale of the polarographic experiments. Such an irreversibility would be expected because of protein adsorption. The useful limit of polarography due to dissolution of mercury from the electrode is only tens of millivolts more positive than the reversible midpoint potential of cytochrome *c*. Thus, were the oxidation of ferrocytochrome *c* as strongly affected by protein adsorption as the reduction of ferricytochrome, then the expected anodic overvoltage of approximately 100 mV would assure that a wave due to oxidation of the ferroheme prosthetic group would be masked by the mercury dissolution from the electrode itself.

The analysis of electrochemical products may be approached from at least two ways. In the controlled potential and dme experiments, the products detected and studied were those stable over periods of several minutes to several hours.

Resolution on a time scale of milliseconds to seconds is possible by generating the reduced protein while simultaneously measuring light absorption.[3] This was accomplished by electrolyzing the cytochrome *c* at a gold mesh electrode, through which the light beam passed. Quantitative experiments are yet to be completed, but observation of the ferroheme absorptions at 425, 520, and 550 nm shows an increase in absorbance initiated by electrolysis. The complementary decrease of the ferriheme band at 530 nm was observed. But the bands centered at 270 and 280 nm, usually assigned to the protein moiety,[10] did not follow the changes in heme absorbance measured in this experiment. These effects may well be due to changes in the protein tertiary structure, and are under further investigation. Reduced material is thus observed as the product of electrochemical reduction, and it continues to be produced as long as electrolysis continues. This supports our previous conclusion that freely diffusing ferrocytochrome *c* is being made at the electrode surface.

## Discussion

The data presented here indicate that the reduction of horse-heart cytochrome *c* at mercury, platinum, and gold electrodes can be accomplished without irreversible protein denaturation. This fact has always been tacitly assumed in potentiometric studies of protein redox systems, and has here been extended to include several nonzero current techniques which may combine control of solution concentrations with measurement of stoichiometry and redox properties. In particular, it is important to find that adsorption of the protein, while present, does not necessarily lead to fouling of the electrode surface and thereby interruption of the usefulness of the electrochemical process. Freely diffusing reduced protein is produced even under circumstances where surface interactions are extensive. The polarographic results clearly show extensive interaction between the protein and the electrode surface; the *i–t* curves are grossly distorted, a low diffusion coefficient is extracted from the polarographic currents, and the observed half-wave potential varies with the protein concentration.

This may be explained in either of two ways, which should be electrochemically equivalent. If the cytochrome *c* must be transported through an adsorbed protein layer in order to be reduced, then the diffusion coefficient observed polarographically should be a function of diffusion through both media and lower than the hydrodynamically obtained value. As an alternative, the slow step in the diffusional mass transport may be a rotational diffusion of adsorbed cytochrome *c* molecules necessary to transfer electrons from the electrode surface to the freely diffusing ferricytochrome *c* molecules. If this latter mechanism has validity, it would be in clear agreement with one possible mechanism for the behavior of cytochrome *c* in the mitochondrial membrane,[30] *i.e.*, wherein the molecule is thought to rotate in order to alternately exchange electrons with the reductase and the oxidase.

The electrochemical studies of metalloporphyrins[31]

(27) C. Greenwood and M. T. Wilson, *Eur. J. Biochem.*, **22**, 5, 11 (1971).

(28) G. H. Czerlinski and K. Dar, *Biochim. Biophys. Acta*, **234**, 57 (1971).

(29) See ref 24, p 562.

(30) B. Chance, C. Lee, L. Mela, and D. DeVault in "Structure and Function of Cytochromes," University Tokyo Press, Tokyo, 1968, p 475.

(31) (a) D. W. Clack and N. S. Hush, *J. Amer. Chem. Soc.*, **87**, 4328 (1965); (b) D. G. Davis and R. F. Martin, *ibid.*, **88**, 1365 (1966); (c) R. Felton and H. Linschitz, *ibid.*, **88**, 1113 (1966); (d) T. M. Bednarski and J. Jordan, *ibid.*, **89**, 1552 (1967); (e) N. E. Tokel, V. Katovic, J. Farmery, L. B. Anderson, and D. H. Busch, *ibid.*, **92**, 400 (1970).

show adsorption complications at least as pronounced as those described here, although their molecular weights are an order of magnitude smaller than cytochrome *c*. A surfactant of more comparable size to cytochrome *c* is Triton X-100, a polyether used by electrochemists to suppress polarographic maxima. This material, when added to a solution containing 5 m*M* Fe(III) and 0.2 *M* sodium oxalate, grossly distorts the iron reduction in 0.005% and completely suppresses normal iron reduction in 0.0125% concentrations.[24] By comparison, cytochrome *c* constitutes a 0.1% concentration in the 100 $\mu M$ solutions described in Tables II and III.

We conclude from these comparisons that the tertiary structure of cytochrome *c* minimizes adsorption to the electrode. Recent studies in this laboratory indicate that when a portion of the protein moiety is removed from cytochrome *c*, some adsorption effects become more severe. Two heme oligopeptides, a nona- and hexadecapeptide,[32] isolated from the native protein show a greatly increased adsorption inhibition of electron transfer,[33] even though the molecular weight of these compounds is approximately one-tenth that of the protein.

Electrochemical studies of protein-free metalloporphyrins and porphyrin-like molecules[12] show convincingly that when reduction occurs at relatively positive potentials, near 0 V, the electron enters an orbital localized predominantly on the metal ion. The first polarographic wave of cytochrome *c* obviously involves iron reduction. A second reduction wave for cytochrome *c* is observed with the reduced protein or at low pH with ferricytochrome. By analogy, this may be due to assumption by the electron of a less stable orbital—perhaps with high electron density on the porphyrin ring. The product of the second electron addition is unstable, since the protein is irreversibly changed when electrolyzed at $-1.0$ V. Addition of a second electron to metalloporphyrins is difficult, not occurring until $\sim -1.0$ V, and it has been postulated that the electron adds to an antibonding orbital of the porphyrin ring.[32c]

The observation of self-inhibition of electron transfer and retardation of mass transport by the protein can be adequately accounted for by at least two different models. Electron transfer through the protein fabric, the Winfield mechanism,[34,35] would seem consistent with an increase in overvoltage with increasing surface coverage while still allowing electron transfer at a protein covered surface. On the other hand, electron transfer primarily at the exposed heme edge[30] is also consistent with adsorption inhibition, provided the edge was alternately to the electrode and freely diffusing exposed protein by rotational diffusion of the surface adsorbed protein.

(32) H. A. Harbury and P. A. Loach, *J. Biol. Chem.*, **235**, 3640 (1960).
(33) M. J. Chang, L. D. Bowers, and L. B. Anderson, unpublished results.
(34) M. E. Winfield, *J. Mol. Biol.*, **12**, 600 (1965).
(35) R. E. Dickerson, T. Takano, O. B. Kallai, and L. Samson, Proceedings of the Wenner-Gren Symposium on Structure and Function of Oxidation Reduction Enzymes, Aug 1970, Stockholm, 1971, in press.

Taken individually, amino acids are not easily reduced electrochemically. None reduce polarographically at less than $-1.0$ V,[29] and none of the individual amino acids with aromatic side chains would be expected to reduce in the potential region accessible to aqueous polarography. Only the metalloporphyrin complex itself in the cytochrome *c* molecule can maintain a significant electron population in thermal equilibrium with an electron donor at $-0.2$ V.

If reduction involves conduction through the protein fabric, then it is nearly certain that transport must be by hole conduction, not electron conduction. It has been reported on the basis of pulse radiolytic studies that the rate of reaction of solvated electrons with the oxidized and reduced forms of cytochrome *c* is rapid and apparently identical.[14] However, these results cannot be readily related to the equilibrium electron transfer properties of the protein. The electron free energy of the solvated electron is equivalent to an electron delivered from an electrode at a controlled potential of approximately $-2.8$ V *vs.* nhe.[36] This energy is sufficient to produce a radical anion from practically any aromatic group on the protein, and the number of possible sites to which the solvated electron may attach is vastly larger than the probable transfer sites for an electron transferred from an electrode poised at $-0.2$ V *vs.* nhe (the equilibrium potential of the cytochrome *c* couple) or transferred from the reductase complex on the mitochondrial surface. On a purely electrochemical basis, it would appear reasonable that electron transfer takes place at the partially exposed heme edge. The cycle of electrochemical reduction could then be completed either by desorption of the reduced protein and approach and adsorption of the next molecule of oxidized protein or by rotational diffusion of the protein. The film of protein thus maintained would be expected to insulate the electrode from interaction with protein molecules beyond this first layer. Inhibition of diffusional mass transport to the electrode surface must be an integral part of the reduction mechanism of cytochrome *c* because the calculated rate of diffusion is clearly lower than expected and is also a function of pH.

Unique mechanisms for maintaining electron conduction through the layer(s) of adsorbed protein must be present, however. Either the compact globular structure of the protein minimizes adsorption and facilitates displacement of reduced by incoming oxidized molecules, or electron transfer through the surface protein layer is possible by conduction or rotational diffusion. No electrochemical activity similar to this is observed in studies involving other strong surfactants.[24]

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