

Rate Constants and Free-Energy Plot for the Reduction of Horse-Heart Cytochrome *c*(III) by Eight Organic Radicals

Christopher D. Borman,[†] A. Mark Dobbing,[†] G. Arthur Salmon,[‡] and A. Geoffrey Sykes^{*,†}

Department of Chemistry, The University of Newcastle, Newcastle upon Tyne, NE1 7RU, U.K., and Cookridge Radiation Research Centre, The University of Leeds, Cookridge Hospital, Leeds LS16 6QB, U.K.

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Rate constants $k_{12}/\text{M}^{-1} \text{s}^{-1}$ (22 °C) for the reduction of the oxidised form of horse-heart cytochrome *c* by eight organic radicals (OR), e.g., methyl viologen $\text{MV}^{\bullet+}$, determined by pulse radiolysis and/or stopped-flow methods, correlate well in a plot of $\log k_{12}$ vs reduction potential E°_1 for the OR. In the pulse radiolysis experiments the ORs were generated in situ by rapid reduction of the parent form by the formate radical $\text{CO}_2^{\bullet-}$, and in the stopped-flow experiments prior reduction of the parent was with dithionite. In both procedures it was necessary to consider formation of double-reduced parent forms. Rate constants k_{12} are in the range 10^9 to 10^4 and E°_1 values from -0.446 to $+0.194\text{V}$. The Marcus free-energy plot of $\log_{10} k_{12} - 0.5 \log_{10} f$ vs $E^\circ_1/0.059$ gives a slope of 0.49, in excellent agreement with the theoretical value of 0.50, with an intercept at $-E^\circ_1/0.059 = 0$ of 6.55 in good agreement with a calculated value of 6.51 from known parameters. The latter includes the reduction potential E°_2 (0.263V) and self-exchange rate constant k_{22} ($0.36 \times 10^3 \text{M}^{-1} \text{s}^{-1}$) for the cytochrome *c*(III)/(II) couple, and assumes a common self-exchange rate constant k_{11} of $1.0 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ for the ORs.

Introduction

Cytochrome *c* is a small structurally well characterised protein containing an Fe–porphyrin prosthetic group (M_r 12.4 kDa; 104 amino acids), which is a component of the mitochondrial electron-transport chain.¹ The aim in the present studies was to determine rate constants (k_{12}) for cross reactions of cytochrome *c*(III) with organic radicals (OR), e.g., $\text{MV}^{\bullet+}$ (1),



and to see whether the k_{12} values conform to the Marcus relationship (2),²

$$k_{12}^2 = k_{11}k_{22}K_{12}f \quad (2)$$

assuming a single common self-exchange rate constant (k_{11}) for the ORs, with K_{12} the equilibrium constant for the cross reaction and k_{22} the self-exchange rate constant for the cytochrome *c*(III)/(II) couple. The term f is defined as in (3)

$$\log f = \frac{(\log K_{12})^2}{4 \log (k_{11}k_{22}/Z^2)} \quad (3)$$

where the collision frequency Z is assumed to be $10^{11} \text{M}^{-1} \text{s}^{-1}$. Using the Nernst equation, $\log K_{12} = (E^\circ_2 - E^\circ_1)/0.059$, with E°_1 and E°_2 reduction potentials in volts (vs NHE) for the parent/OR and cytochrome *c*(III)/(II) couples, respectively, (2) can be expressed as in (4).

$$(\log k_{12} - 0.50 \log f) = 0.50(\log k_{11} + \log k_{22} + E^\circ_2/0.059) - 0.50 E^\circ_1/0.059 \quad (4)$$

This was tested by plotting the left-hand side of (4) against $-E^\circ_1/0.059$, for the eight ORs. Reduction potentials E°_1 for the ORs cover a wide range (-0.446 to $+0.194 \text{V}$) appropriate for reduction of cytochrome *c*(III), $E^\circ_2 = 0.263\text{V}$.³

Electron-transfer reactions of cytochrome *c* have been widely studied^{1,4,5} and in many cases are believed to occur at the solvent accessible edge of the porphyrin.^{6,7} A preference is shown by physiological partners for reaction at an area centred around the point at which the positive end of the dipole axis of cytochrome *c* crosses the surface of the protein. This region is near to the β -carbon of the Phe-82 (midway between Lys-13 and Lys-72) and incorporates part of the porphyrin exposed edge.^{1,6} Reactions of metalloproteins with free radicals have not previously been studied in a systematic manner, and we set out therefore to explore the behavior of eight such radicals with a well characterized protein.

The faster reactions were studied by pulse radiolysis (PR),⁸ with the formate radical $\text{CO}_2^{\bullet-}$ ($E^\circ = -1.9\text{V}$) used to generate the ORs in situ. The slower reactions were studied using the stopped-flow method (SF), with dithionite used to reduce the parent OR's in a prior step. For reactions which give a linear plot of (4) the slope should be equal to 0.50 and the intercept to $0.50 (\log k_{11} + \log k_{22} + E^\circ_2/0.059)$. Related questions are whether the ORs react at the same site on cytochrome *c*, have a common self-exchange rate constant k_{11} ,^{9–11} and whether the charge on the OR (1 + to 2 –) is important.

Experimental Section

Protein. Horse-heart cytochrome *c* from Sigma Chemicals was purified as cytochrome *c*(III) by a procedure already described.¹² Fractions with UV–Vis absorbance (A) ratios A_{550}

[†] Department of Chemistry.

[‡] Cookridge Radiation Research Centre.

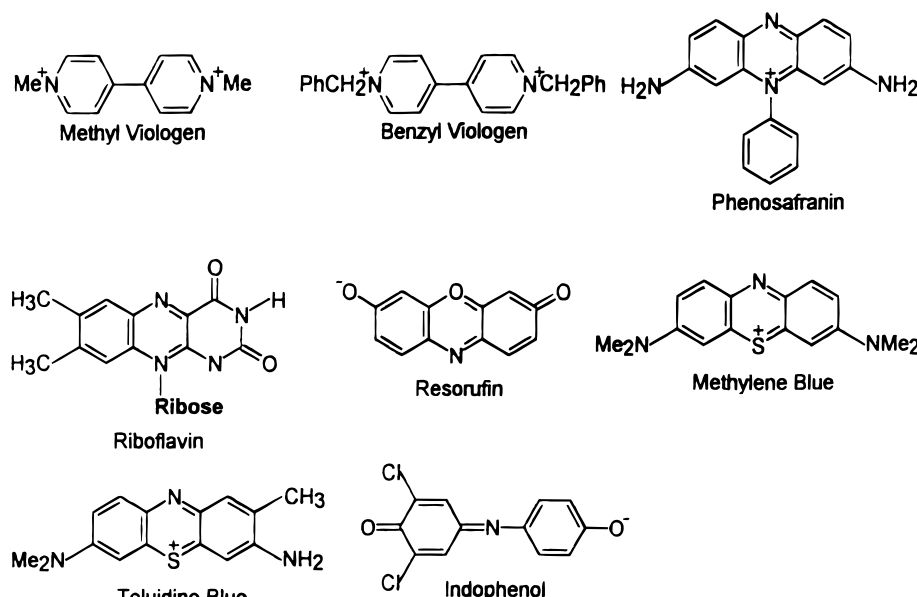


Figure 1. Formulas of eight parent reagents (X) used to generate organic radicals ($X^{\bullet-}$) used in this study.

(reduced) to A_{280} (oxidized) > 1.15 were used for the kinetic studies described. Concentrations were determined from the absorbance at 416 nm ($1.29 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) for reduced protein, with $\Delta\epsilon$ (reduced $>$ oxidized) of $4.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 416 nm.^{1,13}

Reagents for Organic Radicals. The following parent reagents (Figure 1), with trivial, systematic, and abbreviated names as indicated, were used in this work: methyl viologen, $\text{C}_{12}\text{H}_{14}\text{N}_2\text{Cl}_2$ (1,1'-dimethyl-4,4'-bipyridinium dichloride), MV^{2+} ; benzyl viologen, $\text{C}_{24}\text{H}_{22}\text{N}_2\text{Cl}_2$, (1,1'-dibenzyl-4,4'-bipyridinium dichloride), BV^{2+} ; phenosafranin, $\text{C}_{18}\text{H}_{15}\text{N}_4\text{Cl}$ (3,7-diamino-5-phenylphenazinium chloride), Pf^+ ; riboflavin $\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_6$ (vitamin B_2), Rb ; Resorufin, $\text{C}_{12}\text{H}_6\text{NO}_3\text{Na}$, (7-hydroxy-3H-phenoxazine-3-one sodium salt), Rf^- ; methylene blue, $\text{C}_{16}\text{H}_{18}\text{N}_3\text{S}\text{Cl}$ (3,7-bis(dimethylamino)phenazothionium chloride), MB^+ ; toluidine blue ($\text{C}_{15}\text{H}_6\text{N}_3\text{S}\text{Cl}$) $_2\text{ZnCl}_2$ (approximate formula only), TB^+ ; and indophenol $\text{C}_{12}\text{H}_6\text{Cl}_2\text{NO}_2\text{Na}$ (phenolindo-2,6-dichlorophenol sodium salt), IP^- . These were obtained from Sigma Chemicals, except MB^+ and IP^- (BDH), and TB^+ (Fluka).

Toluidine blue (1 g) was recrystallized to constant UV–Vis spectrum and reactivity by first dissolving in H_2O (18 mL) at 80 °C. Ethanol (13.5 mL) was added with stirring over ~ 10 min at 0 °C. The solid obtained was filtered off, washed with cold ether, and stored in a desiccator over silica gel (yield 28%). Titration of the organic redox component with dithionite gave 81% reactivity, as required by the formula $(\text{C}_{15}\text{H}_6\text{N}_3\text{S}\text{Cl})_2\text{ZnCl}_2$ indicated. Some difficulties were encountered in dissolving resorufin to the required levels, and stock solutions were prepared by sonicating for 1 h and then filtering through a 2 μm Acrodisc filter (Gelman). Identical UV–Vis spectra were obtained by making up more dilute solutions by weight, which also gave relevant absorption coefficients (ϵ).

All experiments involving ORs were carried out under rigorous O_2 -free conditions using 99.9% N_2 gas.

Electrochemistry. Reduction potentials (E°_1 vs NHE) for the 1-equiv parent/OR couples were checked by cyclic voltammetry (CV) using a Princeton Applied Research model 173 potentiostat. Control of the equipment was by interfacing to an IBM compatible PC with software from EG & G. A three-electrode system consisting of working (glassy carbon or gold), auxiliary (platinum wire), and reference (Ag/AgCl) electrodes was used. The glassy carbon (or gold) electrode was polished using an

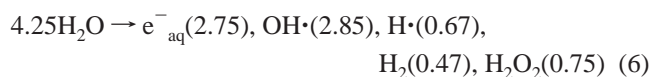
Al_2O_3 slurry (0.30 μm and then 0.015 μm particle size) and Buehler polishing cloth, rinsed with deionized H_2O , and then sonicated in H_2O for ~ 5 min to remove all traces of Al_2O_3 (which can affect readings). Polishing was carried out prior to each scan. The cell was calibrated using the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ couple (0.410 V vs NHE in 0.10 M NaCl). Concentrations of OR parent used were in the 0.1–10.0 mM range, with pH 7.0 (45 mM phosphate), $I = 0.100\text{M}$, and the temperature ~ 20 °C. Measured E° values from CVs were converted to E°_1 values vs normal hydrogen electrode (NHE) by adding 0.222 V to the value determined vs Ag/AgCl .

With two exceptions E°_1 values were within 10 mV of the literature values,¹⁴ see values included in Table 4 below. One exception is with toluidine blue, which gave a value 0.015 mV as compared to a literature value of 0.034 V.¹⁵ With indophenol¹⁶ a 23 mV smaller value was observed. In the case of methylene blue it was necessary to use concentrations at the lower end of the range (0.10 mM) to obtain symmetrical cyclic voltammograms. Under these conditions a value E°_1 of 0.004 V was obtained, as compared to a literature value of 0.011 V.¹⁴ Formation of the double-reduced form probably accounts for the unsymmetrical voltammograms.^{17,18}

Procedure for Pulse Radiolysis. Experiments were carried out on a van de Graaff accelerator at the Cookridge Radiation Research Centre, University of Leeds, using a triple-pass cell (6.9 cm light pathlength) and a 2.5 MeV ($\sim 4 \times 10^{-16}$ J) beam of electrons.¹¹ Pulse lengths were 0.6 μs , and the yield of reducing radicals R for a given pulse is described by (5).

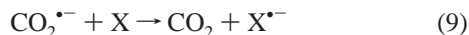
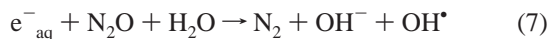
$$[\text{R}] = V_{\text{sec}} S_{\text{sec}} G_{\text{r}} \quad (5)$$

The secondary emission chamber voltage V_{sec} (units V) was measured directly, and the sensitivity S_{sec} (units GyV^{-1}) was obtained by standard thiocyanate dosimetry.¹⁹ The chemical yield of radicals per joule of energy absorbed by the system is G_{r} (units mol J^{-1}). Under the conditions adopted the production of radicals by each pulse was as in (6),

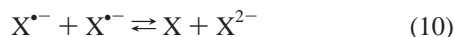


where 10^7 G values in parentheses correspond to the number

of moles of product per joule of energy absorbed. Solutions contained sodium formate (0.010 M) were buffered with phosphate (40.5 mM) and saturated with N₂O, *I* = 0.100 M. Subsequent reactions are (7)–(9)

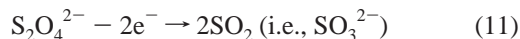


where X^{•−} in the present case is the OR and X the parent form. A large excess of X (1.0 × 10^{−4} M) was used so that the formate radical CO₂^{•−} is effectively scavenged, generating OR (X^{•−}) as the only reducing species present in solution. With such a choice of reactant concentrations double reduction of the parent is precluded, but disproportionation of X^{•−} as in (10),



needs to be considered. Experiments were in the temperature range 22 ± 1 °C.

Stoichiometries of Parent Forms with Dithionite. Prior to SF experiments stoichiometries were determined for the reactions of the four colored parent forms Rf[−], MB⁺, TB⁺, and IP[−] (~30 μM) by titration with sodium dithionite Na₂S₂O₄ (Sigma; ~2 mM) in a glove box (O₂ levels < 3 ppm). Dithionite is a 2 equiv reductant (11).



Solutions of Na₂S₂O₄ were made up by weight, and the purity determined as 84% by spectrophotometric titration with excess [Fe(CN)₆]^{3−} peak at 420 nm (ε = 1010 M^{−1} cm^{−1}). Reactions were rapid (~1 min), and the number of moles of S₂O₄^{2−} required to bring about bleaching of 1 mole of parent were close to 1.0 for the following: Rf[−] (0.96), MB⁺ (0.98), TB⁺ (0.96). In the case of IP[−], which has a quinone type structure, only 0.47 mol were required. Therefore, in the first three cases double reduction of the parent OR is occurring, and the consequences of this need to be considered in the SF experiments.

Procedure for Stopped-Flow. An Applied Photophysics UV–Vis stopped-flow spectrophotometer was used to monitor reactions. Rigorous O₂-free conditions were achieved by replacing connecting leads on the stopped-flow by polyetheretherketone (PEEK) tubing, which has low O₂ permeability, and by prior bubbling of N₂ through reactant solutions. The cytochrome *c*(III) oxidant was present in excess. Reduction of the four parents Rf[−], MB⁺, TB⁺, and IP[−] by dropwise addition of dithionite (~10 mM)²⁰ was carried out using a Gilson pipette-man in the glove box until only a faint color of the parent remained. Excess dithionite was avoided because it is able to reduce cytochrome *c*(III) directly.²¹ The stoichiometry experiments have indicated that under the conditions adopted, formation of the double-reduced products of Rf[−], MB⁺, and TB⁺ occurs and approaches completion. In the case of IP[−], however, the singly-reduced product is obtained. Reactions were monitored at the peak positions λ/nm of the parent forms for Rf[−] (570), MB⁺ (660), TB⁺ (632), and IP[−] (603). At wavelengths > 600 nm, the ε/M^{−1} cm^{−1} for cytochrome *c*(III) (<1500) and for cytochrome *c*(II) (<350) are negligible. At 570 nm, relevant ε/M^{−1} cm^{−1} values are for cytochrome *c*(III) (~5000) and for cytochrome *c*(II) (~500).¹³ In the latter three reactions the formation of cytochrome *c*(II) at 550 nm (Δε = 14.1 × 10³ M^{−1} cm^{−1})¹¹ was also monitored.

Additional information is available from previous PR studies on methylene blue. Thus the OR MB[•] absorbs strongly at 420 nm (ε = 1.04 × 10⁴ M^{−1} cm^{−1}),¹⁷ and the equilibrium constant for (12)



has been determined (~2 × 10⁵), with the forward reaction fast and rate constant in the range (1.5–3.0) × 10⁹ M^{−1} s^{−1}.¹⁷ Experiments with methylene blue were therefore carried out to check the procedure used. Firstly, stopped-flow scan spectra indicated the reappearance of the double-peak feature of parent MB⁺, and the concurrent formation of cytochrome *c*(II) peaks at 416 nm and in the 500–560 nm range in the single-phase process was monitored.¹³ Secondly, sequential stopped-flow mixing of dithionite with MB⁺ (both 7.7 μM), followed by mixing with cytochrome *c*(III) (7.7 μM) after a 2 s delay, gave the same absorbance changes and rate constant as when MB⁺ reduction was carried out in the glove box (which was the preferred method). Finally, stopped-flow mixing of cytochrome *c*(III) (10.6 μM) with MB⁺ (5.3 μM), to which excess dithionite (13.8 μM) had been added, gave a 36% smaller rate constant from absorbance changes at 660 nm. This is accounted for by the reaction of cytochrome *c*(III) with excess dithionite, rate law *k*_{obs} = 1450 [S₂O₄^{2−}]^{1/2} + 1.5 × 10⁴ [S₂O₄^{2−}] as reported by Lambert and Palmer.²¹

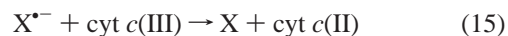
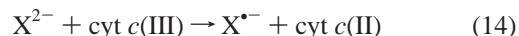
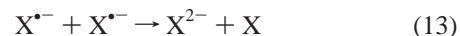
The least favored reaction is that of IP[−] with cytochrome *c*(III), for which reduction potentials give an equilibrium constant of 6.0.

Experiments were in 45 mM phosphate buffer at pH 7.0, *I* = 0.100 M and at 22.0 ± 0.1 °C to correspond with the PR studies.

Treatment of Data. In pulse radiolysis studies the program FACSIMILE²⁰ was used to fit UV–Vis absorbance–time data. In the stopped-flow studies the Applied Photophysics global kinetic analysis program Glint (version 4.10) was used.

Results and Discussion

Pulse Radiolysis Studies. Cytochrome *c*(III) concentrations were in the range 6.0–28.0 μM, with parent levels ~1.0 × 10^{−4} M. On pulsing solutions in situ, OR concentrations < 0.6 μM were generated. The parent forms MV²⁺ and BV²⁺ are colorless, and the radicals MV^{•+} and BV^{•+} have peaks λ/nm (ε/M^{−1} cm^{−1}) at 606(12 400) and 555(11 250), respectively.^{23,24} The following reactions of the radicals, referred to here as X^{•−}, were considered (13)–(15),



here X^{2−} is the double-reduced form. At pH 7.4 reduction potentials for the stepwise conversions MV²⁺ → MV^{•+} → MV⁰ are −0.440 and −0.800V respectively, and disproportionation in (13) is unfavorable and relatively slow.^{25–26} With BV²⁺ dimerization rather than disproportionation in (13) needs to be considered.²⁷ At the concentrations of X^{•−} generated however dimerization is unfavorable. Reaction (15) is therefore dominant in both cases. In the P[•] reaction, the decay of cytochrome *c*(III) at 360 nm (Δε 1.5 × 10⁴), and for Rb^{•−}, the formation of cytochrome *c*(II) at the Soret peak position at 416 nm (Δε = 4.0 × 10⁴ M^{−1} cm^{−1}) were monitored.¹³ No contributions from (14) as a sequel to (13) were apparent in the fitting procedure used. First-order rate constants (*k*_{obs}) for (15) were average

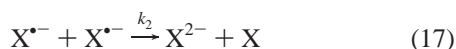
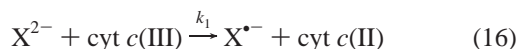
TABLE 1: Rate Constants k_{12} (22 °C) from Pulse Radiolysis Experiments with Cytochrome *c*(III) in the Range (5.0–28.0) $\times 10^{-6}$ M and Parent Organic Compound Present in Large Excess $\sim 1.0 \times 10^{-4}$ M^a

organic radical	$10^6[\text{cyt } c(\text{III})]/\text{M}$	$10^{-3} k_{\text{obs}}/\text{s}^{-1}$	$k_{12}/\text{M}^{-1} \text{s}^{-1}$
$\text{MV}^{\bullet+}$	5.0	3.7	6.7×10^8
	8.0	4.6	
	14.0	8.8	
$\text{BV}^{\bullet+}$	6.0	2.55	4.0×10^8
	10.0	4.0	
	14.0	5.2	
Pf^{\bullet}	7.0	0.37	4.9×10^7
	10.0	0.45	
	18.2	0.90	
	28.0	1.45	
Rb^{\bullet}	6.0	0.258	4.1×10^7
	9.0	0.38	
	12.0	0.48	

^aOrganic radical (OR) concentrations generated were $<0.6 \mu\text{M}$. The pH was 7.0 (40.5 mM phosphate), sodium formate 0.010 M, $I = 0.100$ M.

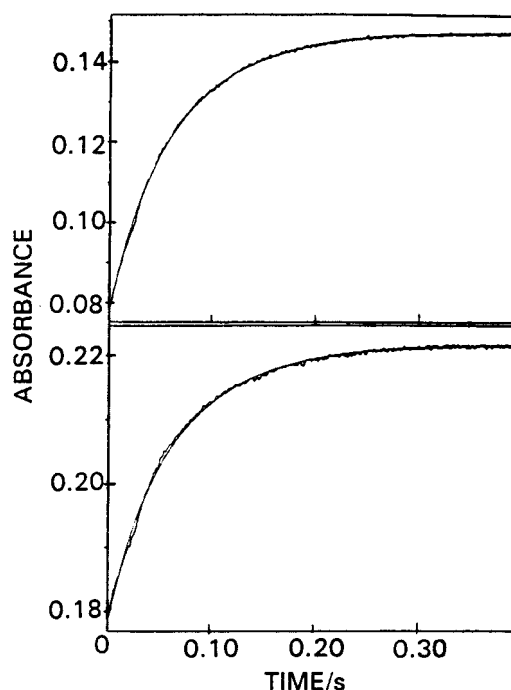
values using in each case 3–4 fresh amounts of reactant solutions, Table 1. Second-order rate constants (k_{12}) for each OR were obtained by an unweighted least-squares on a plot of k_{obs} against $[\text{cyt } c(\text{III})]$. The rate constant k_{12} (25 °C) for $\text{Rb}^{\bullet-}$ reduction of horse-heart cytochrome *c*(III) has been determined previously²⁸ as $6.2 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ at pH = 7.0 (0.10 M phosphate, ionic strength 0.22 M), in satisfactory agreement with the value in Table 1.

Stopped-Flow Studies. Cytochrome *c*(III) concentrations were in excess of the parent form, which was reduced with dithionite prior to SF mixing. In three cases (with $\text{Rf}^{\bullet-}$, MB^+ , and TB^+) dithionite reduces the parent to the double-reduced form. Reformation of the parent, evidenced by peak positions λ/nm ($\epsilon/\text{M}^{-1} \text{cm}^{-1}$) $\text{Rf}^{\bullet-}$ 570 (4.5×10^4), MB^+ 660 (7.8×10^4), TB^+ 632 (5.8×10^4), was observed. To further validate the approach cytochrome *c*(II) formation at 550 nm was also monitored ($\Delta\epsilon = 14.1 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$).¹³ Relevant reactions are indicated in (16)–(18).



The program Glint gave satisfactory fits to (16)–(18) using known $k_2/\text{M}^{-1} \text{s}^{-1}$ values for MB^+ (1.5×10^9) and $\text{Rf}^{\bullet-}$ (3.0×10^8). The former is at the lower end of the range reported,¹⁷ and gives the best fit with smallest errors, e.g., Figure 2.¹⁷ The $\text{Rf}^{\bullet-}$ value was determined directly by PR studies reported elsewhere.²⁸ In the case of TB^+ a k_2 value of $1.5 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ (the same as for MB^+) also gave the best fit, see Table 2. Second-order rate constants obtained are listed in Table 3. Average values of k_3 are listed as k_{12} in Table 4. The similarity of k_1 and k_3 values is noted in Table 3. Similar k_1 ($6.2 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$) and k_3 ($3.1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$) have been reported previously for the reaction of riboflavin $\text{Rb}^{\bullet-}$ with cytochrome *c*(III),²⁹ and for the reaction of five other flavins. With $\text{IP}^{\bullet-}$, parent peak at 603 (1.4×10^4), the double-reduced species is not formed in the reaction with dithionite. Reaction (18) only was considered therefore, and bimolecular plots gave k_3 directly. Average k_3 values are again listed as k_{12} in Table 4.

Free-Energy Plot. Rate constants k_{12} from the PR and SF studies alongside E°_1 values are summarized in Table 4. From

**Figure 2.** Fit of absorbance changes for the reduction of cytochrome *c*(III) (12.9 μM) by MB^+ (2.2 μM) prior to being reduced with dithionite: (a) for the reformation of MB^+ at 660 nm and (b) for the formation of cytochrome *c*(II) at 550 nm. Temperature 22 °C, pH 7.0 (45 mM phosphate), $I = 0.100$ M.**TABLE 2: Analysis of Stopped-Flow Data (22 °C) for the Reduction of Cytochrome *c*(III) (19.8 μM) with TB^+ (1.9 μM) prior Reduced with Dithionite, pH 7.0 (45 mM Phosphate)^a**

$k_1/\text{M}^{-1} \text{s}^{-1}$	$k_2/\text{M}^{-1} \text{s}^{-1}$	$k_3/\text{M}^{-1} \text{s}^{-1}$
$1.2(6) \times 10^6$	$4.3(5) \times 10^7$ ^b	$4.6(8) \times 10^7$
$2.5(1) \times 10^6$	$1.6(1) \times 10^9$ ^c	$3.9(2) \times 10^6$
$2.3(2) \times 10^6$	1.5×10^9	$3.6(2) \times 10^6$

^aTypical fits of k_1 , k_2 , and k_3 (16)–(18) using the Glint program are indicated. In two cases start values were entered for k_2 , and the best fit obtained by an iterative process. The case with k_2 fixed at $1.5 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ gave the best fit. No fit was achieved at k_2 values $< 1.0 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$. ^bStart value $1.0 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$. ^cStart value $3.0 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$.

these it is possible to proceed with the Marcus plot of ($\log k_{12} - 0.50 \log f$) against $-E^\circ_1/0.059$, Figure 3. This is essentially a plot of activation free energies ΔG^\ddagger_2 vs ΔG°_{12} , or more precisely one variable component (E°_1) of ΔG°_{12} . Values of f were calculated from (3) using known parameters. These include k_{22} for cytochrome *c*(III)/(II) electron self-exchange ($0.36 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$),^{1b} and E°_2 for the cytochrome *c*(III)/(II) couple (0.263V).⁸ The correlation obtained, Figure 3, requires that the ORs have very similar electron-self-exchange rate constants (k_{11}). Although k_{11} has not been measured directly, values close to $10^6 \text{ M}^{-1} \text{s}^{-1}$ seem likely.³ Thus in the case of a series of bipyridinium couples of varying potential including $\text{MV}^{2+}/\text{MV}^{\bullet+}$, an estimate of $8.4 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ has been made for reactions in acetonitrile.⁶ On the basis of comparisons involving the $\text{MV}^{2+}/\text{MV}^{\bullet+}$ couple a value $8 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ is reasonable.³ From other information on the reactions of bipyridinium couples with dithionite a value of $1.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ seems most appropriate, with an upper limit of $10^8 \text{ M}^{-1} \text{s}^{-1}$ indicated.⁷

Using an unweighted least-squares treatment of data in Figure 3, the slope of 0.49(2) is in excellent agreement with the theoretical value of 0.50. The intercept 6.55(11) at $E^\circ_1/0.059 = 0$ is also of interest. From (3) this corresponds to 0.50 (log

TABLE 3: Rate Constant Fits to (16)–(18) for the Reduction of Cytochrome c(III) with Parent Forms Reduced with Dithionite Prior to Stopped-Flow Studies^a

λ/nm	[parent]/ μM	[Cyt c(III)]/ μM	$k_1/\text{M}^{-1}\text{s}^{-1}$	$k_2/\text{M}^{-1}\text{s}^{-1}$	$k_3/\text{M}^{-1}\text{s}^{-1}$
Resorufin					
570	0.9	12.3	$5.6(3) \times 10^6$	3.0×10^8	$9.6(4) \times 10^6$
570	2.0	11.4	$5.1(2) \times 10^6$		$13.0(5) \times 10^6$
570	3.4	12.3	$5.0(2) \times 10^6$		$11.0(3) \times 10^6$
Methylene Blue					
660	2.2	12.9	$2.7(1) \times 10^6$	1.5×10^9	$4.4(2) \times 10^6$
550			$2.6(1) \times 10^6$		$4.6(3) \times 10^6$
660	2.3	13.9	$1.8(1) \times 10^6$		$4.2(2) \times 10^6$
660	2.4	23.4	$2.7(1) \times 10^6$		$4.3(2) \times 10^6$
550			$2.6(1) \times 10^8$		$4.3(2) \times 10^6$
Toluidine Blue					
632	1.9	19.8	$2.3(2) \times 10^5$	1.5×10^9	$3.8(2) \times 10^6$
632	2.5	13.7	$2.7(1) \times 10^5$		$3.6(2) \times 10^6$
550			$2.5(1) \times 10^5$		$3.4(3) \times 10^6$
632	3.5	11.1	$2.5(1) \times 10^6$		$3.9(2) \times 10^6$
Indophenol					
603	1.5	16.1			$2.1(2) \times 10^4$
550					$1.9(3) \times 10^4$
603	2.4	11.2			$2.0(1) \times 10^4$
550					$1.9(2) \times 10^4$
603	7.2	14.5			$2.3(2) \times 10^4$

^a Fits were carried out using the Glint program with starting k_2 values as previously determined (see text), or according to the best fit in Table 2.

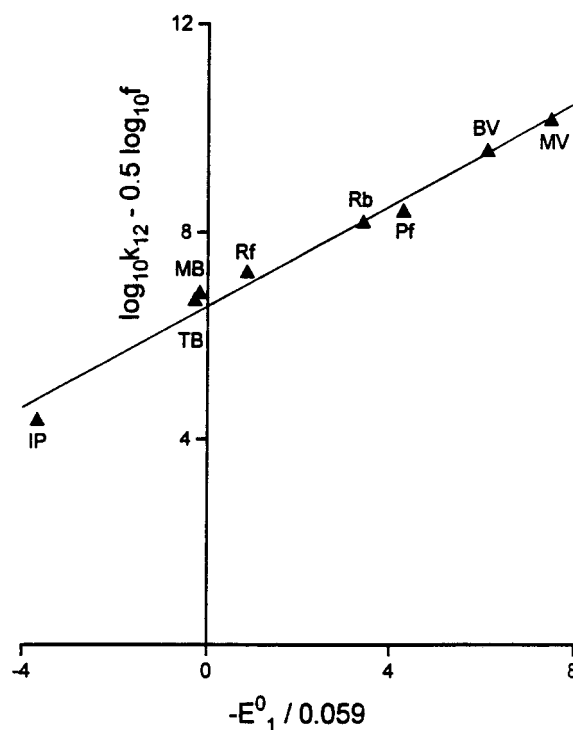
TABLE 4: Listing of Rate Constants $\log_{10} k_{12}$ (22 °C) for Cross Reactions Involving the Reduction of Cytochrome c(III) by Organic Radicals (OR), at pH 7.0, $I = 0.100\text{ M}$, as Determined by Pulse Radiolysis (PR) and Stopped-Flow (SF) Spectrophotometry^a

OR	E°_1/V^b	$\log_{10} k_{12}^c$	$\log_{10} f$
MV ^{•+}	−0.446 (−0.440)	8.82 (PR)	−2.64
BV ^{•+}	−0.355 (−0.359)	8.60 (PR)	−2.07
Pf [•]	−0.243 (−0.252)	7.69 (PR)	−1.42
Rb ^{•−}	−0.200 (−0.208)	7.62 (PR) ^c	−1.15
Rf ^{2−}	−0.059 (−0.051)	7.05 (SF)	−0.53
MB [•]	+0.006 (+0.011)	6.64 (SF)	−0.34
TB [•]	+0.015 (+0.034)	6.57 (SF)	−0.10
IP ^{2−}	+0.194 (+0.217)	4.32 (SF)	−0.01

^a Reduction potentials E°_1 vs NHE and $\log_{10} f$ values from (3) are also listed. ^b Literature values (refs 12–14) are indicated in parentheses. 6.94 by SF method, but only last 20% of reaction monitored.

$k_{11} + \log k_{22} + E^\circ_2/0.059$). With $k_{11} = 1.0 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$, $k_{22} = 0.36 \times 10^3\text{ M}^{-1}\text{ s}^{-1}$, and $E^\circ_2 = 0.263\text{ V}$ for horse-heart cytochrome *c*, a value of 6.51 is obtained in very good agreement with the value from Figure 3. An alternative self-exchange $k_{22} = 1.2 \times 10^3\text{ M}^{-1}\text{ s}^{-1}$ from NMR studies on horse-heart cytochrome *c*,³⁰ increases the intercept to 6.77, and a similar spread of k_{12} values is clearly within the tolerance range of experimental points in Figure 3. No allowance was made for the effect of work terms on k_{22} .

Conclusions. The assumption that all eight ORs have a similar self-exchange rate constant k_{11} close to $1.0 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$ appears to be validated by this work. Adherence to the Marcus equations carries the implication that the mechanism of the electron transfer occurs in the outer sphere and that the ORs react at the same site on the surface of cytochrome *c* for which the same k_{22} applies. In view of the favorable ET rates π – π stacking with an aromatic residue on the cytochrome *c* and/or the exposed heme edge seem plausible modes of interaction. As already indicated the exposed edge of the porphyrin is extensively used for electron transfer. Other sites can be used when special conditions hold and the driving force

**Figure 3.** Marcus free-energy plot for the reduction of cytochrome c(III) with eight organic radicals (OR) as listed in Table 4.

is favorable.³¹ The correlation applies over a wide range of E°_1 values from −0.446 to +0.194V, with rate constants in the range 10^9 to $10^4\text{ M}^{-1}\text{ s}^{-1}$. No dependence on charge of the OR is apparent in Figure 3, suggesting that the effect of electrostatic charge is minimal at the reaction site used on cytochrome *c*. However acid–base properties of OR have not been taken into account and the charges indicated in the first column of Table 4 may need amending. For example riboflavin at pH 7.0 is present largely as $\text{H}^+\text{Rb}^{•−}$ (pK_a 8.3).³²

With the successful experience gained from this study it is possible to explore other less well understood metalloproteins, including multisite proteins,²⁸ using the same eight ORs with a self-exchange rate constant of $1.0 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$.

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