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# REDUCTION OF FERRICYTOCHROME *c* BY HYDROGEN ATOMS

## EVIDENCE FOR INTRAMOLECULAR TRANSFER OF REDUCING EQUIVALENT*

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## SUMMARY

Direct evidence obtained by means of the technique of pulse radiolysis–kinetic spectrometry, with measurements in the time range $10^{-6}$ to 1 s, is presented that, consequent upon reaction of a single H-atom with a single molecule of ferricytochrome *c*, a reducing equivalent is transmitted via the protein structure to the ferriheme moiety. Such transmission accounts for at least 70% of the total reduction of the ferri to the ferro state of cytochrome *c*. The remainder of the total reduction takes place without stages resolvable on the time scale of these experiments. Reduction brought about by H atoms appears to follow a different course than reduction by hydrated electrons. In the latter case, intramolecular transmission of reducing equivalents could not be demonstrated (Lichtin, N. N., Shafferman, A. and Stein, G. (1973) Biochim. Biophys. Acta 314, 117–135).

Not every H-atom reacts with ferricytochrome *c* at a site which results in conversion of the Fe(III) state to the Fe(II) state. Approximately half of reacting H-atoms do not produce reduction.

The following second order rate constants have been determined in solutions of low ionic strength at 20±2 °C: $k$[H+ferricytochrome *c*] = (1.0±0.2) · $10^{10}$ $M^{-1} \cdot s^{-1}$ at pH 3.0 and 6.7; $k$[H+ferrocytochrome *c*] = (1.3±0.2) · $10^{10}$ $M^{-1} \cdot s^{-1}$ at pH 3.0; $k[e^-_{aq}$+ferrocytochrome *c*] = (1.9±0.4) · $10^{10}$ $M^{-1} \cdot s^{-1}$ at pH 6.7.

## INTRODUCTION

The first paper [1] of this series reported a study of the reduction of dilute aqueous ferricytochrome by the hydrated electron by means of the technique of pulse radiolysis–kinetic spectrometry. In this work the first spectrum resolvable by our technique generated by bimolecular reaction of an electron with a molecule of

* Paper II in the series "Reaction of cytochrome *c* with one electron redox reagents".

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ferricytochrome *c*, $k$[ferricytochrome $c+e^-_{aq}$] = $(6.0\pm0.9)\cdot10^{10}\ M^{-1}\cdot s^{-1}$ in neutral salt free solution at 20 °C, was assigned to unrelaxed ferrocytochrome *c*. Subsequent first order intramolecular processes, $k[p \rightarrow s] = (1.2\pm0.1)\cdot10^5\ s^{-1}$ and $k[s \rightarrow t] = (1.3\pm0.2)\cdot10^2\ s^{-1}$, were ascribed to relaxation of the tertiary structure of the protein to the equilibrium conformation of ferrocytochrome *c*. No spectral transformation was observed which could be ascribed with certainty to intramolecular transmission of reducing equivalent from a site of initial addition of an electron to ferriheme. Our first preliminary pulse radiolytic investigation [2] of the reaction of H-atoms with ferricytochrome *c* had, in contrast, indicated such intramolecular transmission. We can now report the confirmation of these indications.

We have previously described in some detail the pulse radiolytic conditions under which the reaction of one molecule of reducing agent, e.g. H-atom or $e^-_{aq}$, with one molecule of ferricytochrome *c* can be studied [1]. These conditions were employed in the present work. In particular, H-atoms were isolated from the reactive species $e^-_{aq}$ and $\dot{O}H$ produced by radiolysis of water either by conversion of hydrated electrons to H-atoms by reaction with $H^+$ or via conversion to $\dot{O}H$ radicals by reaction with $N_2O$ and elimination of $\dot{O}H$ by reaction with *tert*-butanol. This yields $\dot{C}H_2C(CH_3)_2OH$ radicals which have been shown to be unreactive with respect to ferricytochrome *c* [3]:

$$e^-_{aq}+H^+ \rightarrow H \quad k = 2.3\cdot10^{10}\ M^{-1}\cdot s^{-1}\ [4] \tag{1}$$

$$e^-_{aq}+N_2O_{aq} \rightarrow \dot{O}H+N_2+OH^- \quad k = 5.6\cdot10^9\ M^{-1}\cdot s^{-1}\ [4] \tag{2}$$

$$\dot{O}H+(CH_3)_3COH \rightarrow H_2O+\dot{C}H_2C(CH_3)_2OH \quad k = 5\cdot10^8\ M^{-1}\cdot s^{-1}\ [5] \tag{3}$$

Reactions 1+3 are used conveniently in acid solutions while Reactions 2+3 can be used around neutral pH.

It has been shown previously that H-atoms (generated externally by electric discharge in $H_2$) reduce aqueous ferricytochrome *c* to the native ferroenzyme, as evidenced by absorption spectrum and resistance to autoxidation of the product; aqueous ferrocytochrome *c* is itself degraded by the further action of H-atoms [6]. In the present work such degradation was minimized by exposing each aliquot of ferricytochrome *c* solution to only one pulse and maintaining conditions such that the initial ratio of ferricytochrome *c* to H-atoms was never less than 5 and was usually greater than 10. Resulting changes in optical absorption were determined at times extending from $5\cdot10^{-7}$ s to about 1 s after the pulse. These data have provided information on the kinetics of initial reaction of H-atom with ferricytochrome in dilute salt-free aqueous solution and of subsequent transformations as well as confirmation of spectral data obtained previously with externally generated H-atoms [6].

## EXPERIMENTAL

### *Materials*

Materials were the same as those employed previously [1] with the following exceptions:

Argon (used to deaerate solutions irradiated at acid pH) [1] and $N_2O$ (employed for irradiation near neutrality) [1] were routinely freed of $O_2$ by bubbling

through three traps containing vanadium (II) prepared by in situ reduction of Fluka "purum" grade $NaVO_3$ with zinc amalgam prepared from B.D.H. "Analytical Reagent" zinc and Frutarom "Analytical" grade mercury. Aqueous solutions of ferricytochrome *c* [1] were prepared no more than 6 h before use and their pH adjusted by titration with Baker "Analyzed Reagent" $HClO_4$. Ferrocytochrome *c* was prepared by reduction of ferricytochrome *c* [1] with $Na_2S_2O_4$ (Riedel-DeHaën) or hydrogen (Matheson) over palladium black (Fluka, Puriss.). In the former procedure, approx. $10^{-4}$ M ferricytochrome *c* was treated with a 5-fold excess of $Na_2S_2O_4$ in 0.005 M phosphate buffer at pH 6.5. The reduced enzyme was separated from inorganic salts by two cycles of adsorption on Sephadex Medium G-25 and elution under $O_2$-free conditions with 0.005 M phosphate buffer. In the latter procedure an approx. $10^{-4}$ M solution of ferricytochrome *c* in 0.005 M pH 6.7 phosphate buffer was deoxygenated by sweeping with $N_2$, a catalytic amount of Pd-black was added and $H_2$ bubbled through the solution at 1 atm until the absorption spectrum of an aliquot indicated 90–95 % completion of reduction. The catalyst was then removed by centrifugation.

*Apparatus and procedures*

Apparatus and procedures were the same as those described previously [1].

*Ratio of reactants*

The concentration of *tert*-butanol was $10^4$ times the concentration of ferricytochrome *c* in all experiments. Assuming that $k[\dot{O}H + \text{ferricytochrome } c]$ is about $6 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$, a reasonable estimate in view of reported specific rates of reaction of $\dot{O}H$ with ribonuclease [7] $(2.4 \pm 0.6) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$, lysozyme [8] $4.9 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ and trypsin [9] $(8.2 \pm 1.2) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$, scavenging of hydroxyl radicals by *tert*-butanol was at least 99 % complete in all cases. As a result, the amount of $\dot{O}H$ available for reaction with ferricytochrome *c* was less than 1 % of available H-atom at acid pH and less than 10 % at neutral pH.

The concentrations of ferricytochrome *c* employed in experiments performed around pH 3 fell in the range $1 \cdot 10^{-5}$–$6 \cdot 10^{-5}$ M. Taken together with the specific rate of Eqn 1 and $k[e^-_{aq} + \text{ferricytochrome } c] = 6.0 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ it follows that for this concentration range the amount of $e^-_{aq}$ available for reaction with ferricytochrome *c* was 3 % to 15 % of available H-atom. Experiments at neutral pH employed $1 \cdot 10^{-5}$–$10 \cdot 10^{-5}$ M ferricytochrome *c* and $2.5 \cdot 10^{-2}$ M $N_2O$. The amount of $e^-_{aq}$ available for reaction with ferricytochrome *c* was, accordingly, 0.4–4 % of available H-atom. Under the conditions employed approx. 8 % of the H-atoms produced in solution reacted with *tert*-butanol [10].

## RESULTS

*Spectra*

At each wavelength measured, absorbance changed continuously and in the same sense from the time of initial observation until about 300 ms after the pulse. Time resolution of different spectral stages was not possible for the reaction of H-atom as it was for that of $e^-_{aq}$. (See Kinetics section below.) No further changes in absorbance were observed later than 500 ms after the pulse. The final spectrum mea-

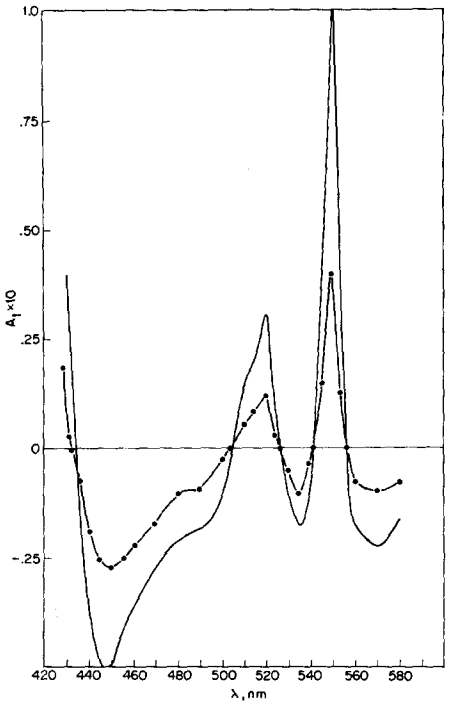


Fig. 1. Spectrum measured 500 ms after application of ~1700 rad pulses, $[H]_0 = 1.4 \cdot 10^{-6}$ M, to $2 \cdot 10^{-5}$ M ferricytochrome *c* at pH 6.7 (●—●) compared with spectrum calculated from the data of [10] for 100% reduction to ferrocytochrome *c* (—); $l = 4$ cm.

sured 500 ms after the pulse is compared in Fig. 1 with a spectrum calculated from the data of Margoliash and Frohwirt [11], assuming 100% reduction of ferricytochrome *c*, by means of Eqn 4,

$$A/l = [\varepsilon_{ferro} - \varepsilon_{ferri}]\,[ferro] \tag{4}$$

where $A/l$ is the absorbance per unit optical pathlength and $\varepsilon_{ferro}$ and $\varepsilon_{ferri}$ are molar absorbance indices. The close similarity of observed and calculated spectra is apparent. Isosbestic points were, in addition, found in separate measurements to coincide within experimental error (±1 nm, Hilger Watts Model D 330/331 double monochromator) with those reported [11] at 339, 434, 504, 542 and 556.5 nm.

Efficiencies of reduction of ferricytochrome *c* to the ferro state were calculated by comparing optical absorbance measured 300 ms after the pulse with absorbances calculated [1, 11] for quantitative reduction. Concentrations of ferrocytochrome *c* employed in the latter calculation were arrived at by allowing for consumption of approx. 8% of H-atoms by *tert*-butanol and by correcting for wastage of some initially produced H-atoms via their combination to give $H_2$. This correction was accomplished by means of Eqn 5*,

* Eqn 5 results from the integration of $-d(H)/dt = k_6[H]\,[FeIIICyt\ c] + 2k_7[H]^2$ to obtain [H] as $f[t, (FeIIICyt\ c)_t]$ followed by substitution of the latter function into $-d[FeIIICyt\ c]/dt = k_6[H]\,[FeIIICyt\ c]$ and integration from $t = 0$ to $t = \infty$.

$$(\text{FeIICyt } c) = [k_6(\text{FeIIICyt } c)_0/2k_7]\ln[1+2k_7(\text{H})_0/k_6(\text{FeIIICyt } c)_0] \quad (5)$$

$$\text{H}+\text{FeIIICyt } c \xrightarrow{k_6} \text{FeIICyt } c \quad (6)$$

$$2\text{H} \xrightarrow{2k_7} \text{H}_2 \quad (7)$$

where $k_6 = 1.0 \cdot 10^1\ \text{M}^{-1} \cdot \text{s}^{-1}$ (see Kinetics section below) and $2k_7 = 2 \cdot 10^{10}\ \text{M}^{-1} \cdot \text{s}^{-1}$ [4]. Resulting values at several wavelengths, concentrations of ferricytochrome *c* and initial concentrations of H-atoms at pH 3.0 and 6.7, respectively, are summarized in Tables I and II. From these data it is apparent that the efficiency of reduction of ferricytochrome *c* to the ferro state by H-atoms is approx. 50%, essentially independent of wavelength of measurement, initial concentration of H-atoms and pH. This result agrees with that previously measured at neutral pH at several wavelengths different from those employed here [1].

Because of the proposed involvement of tyrosines 67 and 74 in the biological transmission of reducing equivalents to heme in the revised version of the mechanism of Winfield [12] advanced by Dickerson et al. [13], especial attention was given to absorbance changes at pH 3.0 (chosen because of greater experimental sensitivity) in the wavelength region 325–350 nm, in the vicinity of the absorption maximum of the cyclohexadienyl radical produced by attachment of an H-atom to the aromatic ring of phenol [14], $\lambda_{max} = 330 \pm 5$ nm, $\varepsilon_{max} = 3800 \pm 800\ \text{M}^{-1} \cdot \text{cm}^{-1}$. Even though this wavelength region includes the ferri–ferro isosbestic point at 339 nm, no absorption changes were observed over the time range $10^{-6}$–$10^{-1}$ s other than those ascribed

TABLE I

REDUCTION YIELDS AT pH 3.0 MEASURED AT VARIOUS WAVELENGTHS

Yield is calculated from measured absorbances and the spectral data of [10] by means of the equation [ferrocytochrome *c*] = $A/l\ [\varepsilon_{ferro} - \varepsilon_{ferri}]$.

| nm | Ferricytochrome *c* concn ($\mu$M) | H concn ($\mu$M) | Yield* (%) |
|---|---|---|---|
| 572 | 10 | 1.6 | 70 |
| 550 | 60 | 4.3 | 68 |
| 550 | 40 | 1.8 | 52 |
| 550 | 40 | 4.3 | 50 |
| 550 | 20 | 1.6 | 70 |
| 550 | 20 | 1.8 | 54 |
| 550 | 20 | 3.8 | 54 |
| 550 | 20 | 4.3 | 45 |
| 550 | 10 | 1.6 | 52 |
| 550 | 10 | 3.8 | 68 |
| 521 | 10 | 1.6 | 48 |
| 450 | 20 | 1.8 | 55 |
| 450 | 20 | 3.7 | 65 |
| 450 | 20 | 4.3 | 70 |
| 426 | 20 | 4.3 | 36 |
| | Average yield = 57±10% | | |

* Each value is the mean of at least two independent measurements. Probable error of measurement, ±10%.

TABLE II

REDUCTION YIELDS AT pH 6.7 MEASURED AT VARIOUS WAVELENGTHS

See Table I for details.

| nm | Ferricytochrome *c* concn ($\mu$M) | $[H]_0$ ($\mu$M) | Yield (%) |
|---|---|---|---|
| 572 | 10 | 2.3 | 52 |
| 550 | 100 | 2.6 | 58 |
| 550 | 60 | 1.4 | 41 |
| 550 | 60 | 2.3 | 50 |
| 550 | 60 | 2.6 | 50 |
| 550 | 40 | 1.2 | 57 |
| 550 | 40 | 2.3 | 50 |
| 550 | 30 | 2.6 | 54 |
| 550 | 20 | 2.3 | 39 |
| 550 | 10 | 2.2 | 43 |
| 521 | 10 | 2.3 | 36 |
| 450 | 30 | 2.6 | 75 |
| 450 | 20 | 2.3 | 43 |
| 450 | 20 | 4.3 | 54 |
| 450 | 10 | 2.3 | 45 |
| 426 | 20 | 4.3 | 40 |
| | Average yield = 49±8 % | | |

to conversion of the spectrum from that of ferricytochrome *c* to that of the ferro form.

*Kinetics of reaction of ferricytochrome c with H-atoms*

Preliminary work had indicated that initial reaction of H-atom with ferricytochrome *c* is followed by intramolecular processes causing spectral transformations in the $\alpha$ and $\beta$ bands, time resolution of which was difficult [2]. These early observations

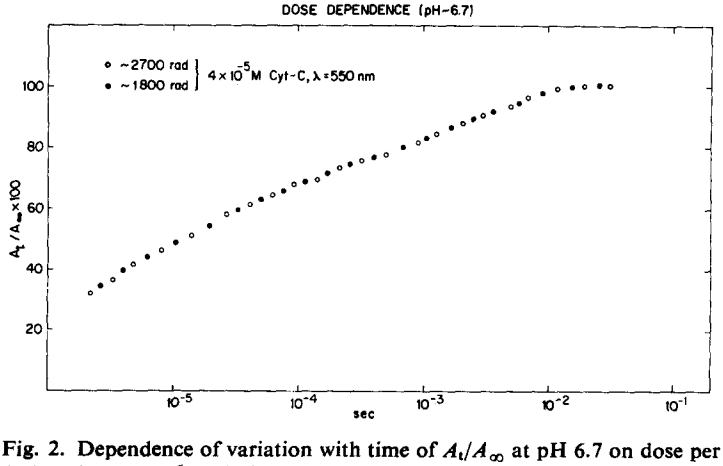


Fig. 2. Dependence of variation with time of $A_t/A_\infty$ at pH 6.7 on dose per pulse. $[H]_0 = 1.5 \cdot 10^{-6}$ (●) and $2.2 \cdot 10^{-6}$ M (○).

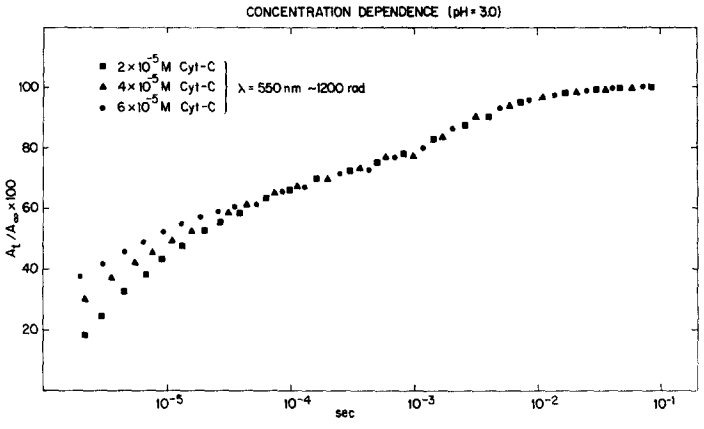


Fig. 3. Dependence of variation with time of $A_t/A_\infty$ at pH 3.0 on concentration of ferricytochrome *c*. $[H]_0 = 6 \cdot 10^{-6}$ M.

are confirmed and extended by the data summarized in Figs 2–6 in which $A_t/A_\infty$, the ratio of absorbance at time $t$ to its final value, is plotted on a linear scale versus time on a logarithmic scale. (This plot should not be confused with a conventional first order plot of log $A_t$ vs $t$.) The spectral changes upon which these figures are based corresponded in sign to those expected from reduction of cytochrome *c* from its ferri to its ferro state [11]. Each of these figures is a composite of the results of more than 30 experiments in each of which an aliquot was exposed to a single pulse. Each point on a plot is the average of four independent measurements. Probable error of a single measurement is estimated as 20 %. Fig. 2 documents that at pH 6.7 absorbance in the α-band changes at a rate which is independent of dose per pulse, i.e. of initial concentration of H-atoms. Virtually identical results were obtained at pH 3.0 on comparing doses per pulse of 350 and 1000 rad delivered to $4 \cdot 10^{-5}$ M ferricytochrome *c*. Figs 3 and 4 demonstrate the effects of variation in initial concentration of ferricyto-

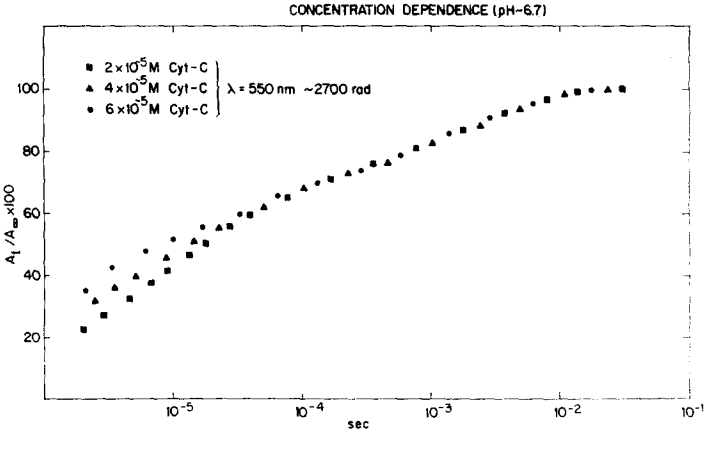


Fig. 4. Same as Fig. 3, pH 6.7. $[H]_0 = 2.2 \cdot 10^{-6}$ M.

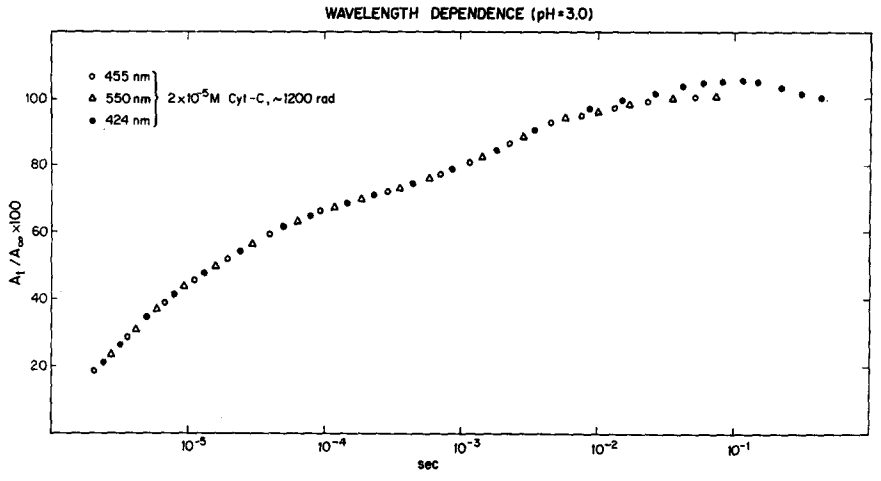


Fig. 5. Dependence of variation with time of $A_t/A_\infty$ at pH 3.0 on wavelength of measurement. $[H]_0 = 6 \cdot 10^{-6}$ M.

chrome *c* at pH 3.0 and 6.7. The dependence of relative absorbance on substrate concentration during the first 50 $\mu$s reflects the addition of H-atoms to ferricytochrome *c*, a second order process. At later stages, relative absorbance increases with time at a rate independent of concentration of substrate. Together, Figs 2–4, establish the intramolecular nature of changes in absorbance in the band taking place after the initial bimolecular reaction of H-atoms with ferricytochrome *c* is essentially complete. Figs 5 and 6 show that the kinetics of spectral change is identical at 424, 455 and 550 nm up to about 0.5 ms after the pulse at pH 6.7 and up to about 5 ms at pH 3.0. At longer times, small differences are apparent at different wavelengths and the nature of these differences is pH dependent. During the periods of wavelengths independence, the spectral changes at different wavelengths apparently reflect the same intramolecular transformation.

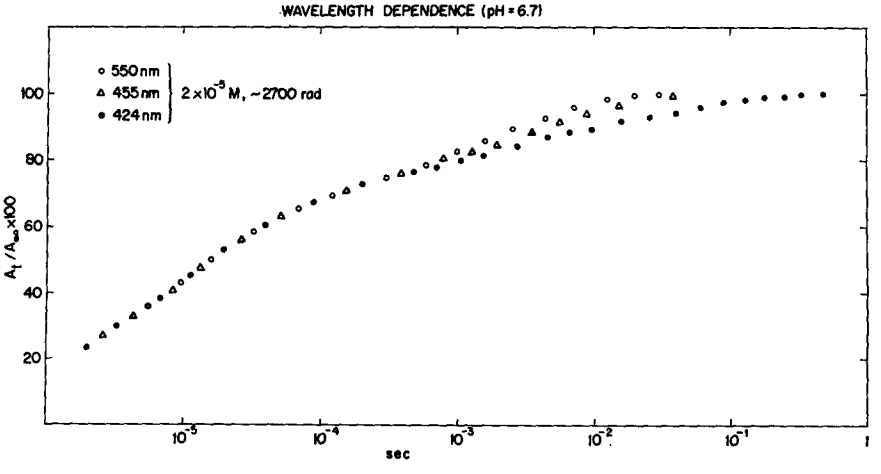


Fig. 6. Same as Fig. 5, pH 6.7. $[H]_0 = 2.2 \cdot 10^{-6}$ M.

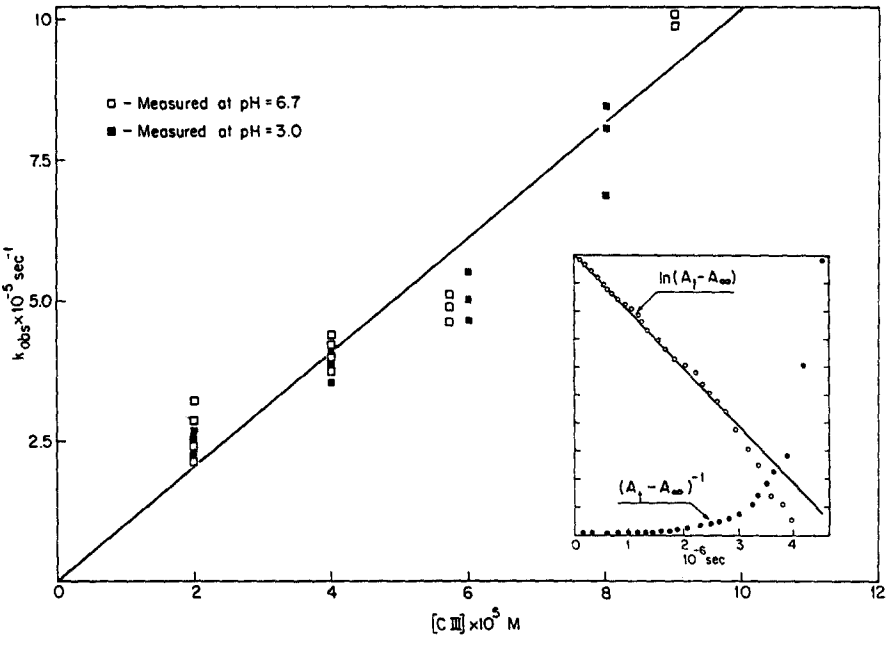


Fig. 7. Observed pseudo first-order specific rates vs [ferricytochrome *c*]. Insert, first and second order plots for a single run.

Calculation of $k$[H+ferricytochrome *c*] is complicated by the overlap of changes in absorbance due to the fast bimolecular reaction of substrate with H-atoms and slower changes due to intramolecular transformations. Such overlap is minimized at relatively high initial concentrations of ferricytochrome *c*: e.g. Fig. 7 insert shows that with $8 \cdot 10^{-5}$ M substrate adherence of the data to (pseudo) first order kinetics in the initial stages of the reaction is not bad while deviation from second order behavior is large. (Pseudo first order behavior is expected since ferricytochrome *c* is in large excess.) In Fig. 7 pseudo first-order rate constants, each of which was calculated from one or more half lives, are plotted against concentration of ferricytochrome *c*. The essentially linear dependence establishes first order dependence on concentration of substrate and, therefore, the second order nature of the initial action of H-atoms on cytochrome *c*. The data of Fig. 7 include measurements employing doses per pulse of 350, 700 and 1000 rad at pH 3.0 and of 1500 and 2700 rad at pH 6.7. Measurements were made at 550 nm for all concentrations of ferricytochrome *c*, at 460 nm with $2.0 \cdot 10^{-5}$ and $4.0 \cdot 10^{-5}$ M substrate solutions and at 426 nm with $2 \cdot 10^{-5}$ M substrate. From these measurements $k$[H+ferricytochrome *c*] $= (1.0 \pm 0.2) \cdot 10^{10}$ M$^{-1} \cdot$ s$^{-1}$ in excellent agreement with previously reported values of $1 \cdot 10^{10}$ M$^{-1} \cdot$ s$^{-1}$ and $(1.2 \pm 0.2) \cdot 10^{10}$ M$^{-1} \cdot$ s$^{-1}$ based on pulse radiolytic data [2, 3] and $1 \cdot 10^{10}$ M$^{-1} \cdot$ s$^{-1}$ based on competition kinetics employing externally generated H-atom [6].

*Kinetics of reaction of ferrocytochrome c with H and* $e^-_{aq}$

Second order specific rates of reaction of ferrocytochrome *c* with H-atoms and, respectively, hydrated electrons were measured so that they could be compared with

the specific rates of the corresponding reactions of the ferri form. These measurements were based on changes in absorption resulting from the action of H-atoms on ferrocytochrome *c* or, in the case of $e^-_{aq}$, on the disappearance of its absorption. The value of $k$[H+ferrocytochrome *c*], $(1.3\pm0.2)\cdot 10^{10}\ M^{-1}\cdot s^{-1}$, at pH 3.0 measured at 330 and 340 nm with $1.0\cdot 10^{-5}$ M and $3.0\cdot 10^{-5}$ M ferrocytochrome *c* and doses of 500 and 1000 rad · pulse$^{-1}$, is almost identical with the specific rate of reaction of H-atoms with the Fe(III) form. In contrast, $k[e^-_{aq}$+ferrocytochrome *c*] is approximately one third the value of $k[e^-_{aq}$+ferricytochrome *c*]. Measurements of the former rate constant were caried out at 600, 640 and 660 nm on $1.0\cdot 10^{-5}$, $2.0\cdot 10^{-5}$ and $3.4\cdot 10^{-5}$ M solutions buffered to pH 6.7 with $5\cdot 10^{-3}$ M $NaH_2PO_4$ using 800 and 1700 radpulse$^{-1}$ · doses. With ferrocytochrome *c* prepared by reduction of the Fe(III) form with dithionite the specific rate of reaction with hydrated electrons was found to be $(1.3\pm0.3)\cdot 10^{10}\ M^{-1}\cdot s^{-1}$ while with ferrocytochrome *c* produced by reduction of the Fe(III) form with $H_2$ over Pd it was $(1.9\pm0.4)\cdot 10^{10}\ M^{-1}\cdot s^{-1}$. These values can be compared with $k[e^-_{aq}$+ferricytochrome *c*$] = (6.0\pm0.9)\cdot 10^{10}\ M^{-1}\cdot s^{-1}$ which we have reported [1] for neutral pH in the absence of added electrolytes and with the value $4.8\cdot 10^{10}\ M^{-1}\cdot s^{-1}$ which we have obtained for this rate constant in the presence of $5\cdot 10^{-3}$ M phosphate buffer.

## DISCUSSION

### *Intramolecular transfer of reducing equivalents*

The possibility of transmission of reducing equivalents through the protein structure to the Fe(III) in the biological reduction of ferricytochrome has been advanced by Winfield [12] and elaborated by Dickerson et al. [13], largely on structural grounds. Direct evidence of such a process has, however, been lacking in in vitro experiments with ferricytochrome *c*. The technique of pulse radiolysis–kinetic spectrometry has revealed rapid intramolecular transmission of radical sites consequent upon the reactions of solvated electrons as well as H-atoms and ·OH radicals with ribonuclease [7, 15]. In the reaction of ferricytochrome *c* with hydrated electrons the occurrence of intramolecular transmission could not be clearly demonstrated [1, 16–18]. Available data do not exclude the possibility of extremely rapid reaction, e.g. [16] $k_{transmission} > 4\cdot 10^6\ s^{-1}$, but they are also consistent with addition of an electron directly to ferriheme. Thus, we have shown in this paper that $k[e^-_{aq}$+ferrocytochrome *c*$] = (1.6\pm0.7)\cdot 10^{10}\ M^{-1}\cdot s^{-1}$ under conditions similar to those under which $k[e^-_{aq}$+ferricytochrome *c*$] = 4.8\cdot 10^{10}\ M^{-1}\cdot s^{-1}$. The specific rate of reaction of the Fe(II) form can be taken as a measure of the specific rate of reaction of hydrated electrons with the protein part of ferricytochrome *c*. These data can be interpreted as indicating that ~67 % of incident electrons react directly with the ferriheme part of the molecule while the remainder is captured by protein sites. The efficiency of reduction which we have recently reported [1], 74±3 %, is then consistent with the assumption that only those electrons which react directly with ferriheme lead to its reduction; transmission of reducing equivalents from primary sites in the protein structure need not be assumed. It should be pointed out here that support claimed for the Winfield–Dickerson mechanism from data based on pulse radiolysis [19] has been inferred from indirect data involving correlation of the pH dependences of the specific rate of reaction of hydrated electrons with ferrinitrocytochrome *c*, absorbance

at 695 nm and the ionization state of the nitrated tyrosyl-67 residue. The same authors also infer [19] from their observation that, in 0.1 M sodium formate at pH 7, $k[e^-_{aq}+$ ferricytochrome $c]$ is virtually identical with $k[e^-_{aq}+$ferriheme] that there is electron transfer from the surface of the ferricytochrome $c$ to the prosthetic group.

There is no evidence for intramolecular transmission of reducing equivalents in the slower reduction of ferricytochrome $c$ by the free radical reducing agents [3] derived from malate, $k = (8.5 \pm 0.8) \cdot 10^7\ M^{-1} \cdot s^{-1}$, lactate, $k = (2.4 \pm 0.2) \cdot 10^8\ M^{-1} \cdot s^{-1}$, and ethanol, $k = (1.8 \pm 0.2) \cdot 10^8\ M^{-1} \cdot s^{-1}$. Efficiency of reduction to ferrocytochrome $c$ by these reagents is 100 %.

The still slower kinetics of reaction of aqueous Cr(II) with ferricytochrome $c$, measured by stopped-flow technique, has been interpreted as involving electron transfer via a Cr–Cl–Fe bridge in the presence of $Cl^-$ and via a "remote" pathway in the presence of iodide, azide or thiocyanate ions [20]. However, the "remote" pathway is visualized in the latter cases to involve bridging via attachment of these ions to porphyrin and does not implicate transmission of reducing equivalents via the surrounding protein.

The reduction of ferricytochrome $c$ by H-atoms stands in contrast to the behavior of hydrated electrons, organic radicals and Cr(II). For H-atoms, the evidence for intramolecular transfer of reducing equivalents to Fe(III) via the protein structure is strong. Intramolecular change in absorbance occurs over the time period corresponding to at least the final 50 % of reduction of ferricytochrome to ferrocytochrome $c$. The magnitudes of relative changes in absorbance are independent of wavelength of measurement except for small differences which appear relatively late and which are pH dependent. Wavelength independence of the magnitude of relative changes in absorbance is expected if the spectral changes reflect only the difference in absorption between relaxed ferricytochrome $c$ and relaxed ferrocytochrome $c$. The small, pH dependent differences which appear at times of the order of $10^{-3}$ s after the pulse can be ascribed to relaxation of the tertiary structure of reduced cytochrome $c$ [1, 21, 22]. It is unlikely that the observed intramolecular transformations involve electron transfer to iron following direct attachment of H-atoms to porphyrin and loss of a proton. The observed kinetics might be interpreted as resulting from attachment of H-atom at different sites in the porphyrin and subsequent loss of protons at different rates from the different sites. Proton loss would have to be the rate determining step in this type of transmission process because subsequent transfer of an electron to iron via $\pi$ orbitals would be many orders of magnitude faster than the fastest part of the observed intramolecular changes. However, the transient radical intermediates resulting from addition of H-atoms to porphyrin which are assumed in this mechanism would have spectra different from those of oxidized or reduced hemin and are therefore inconsistent with the spectral data. Further support for involvement of protein comes from the essentially identical magnitudes of $k[H+$ferricytochrome $c] = (1.0 \pm 0.2) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$, and $k[H+$ferrocytochrome $c] = (1.3 \pm 0.2) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. This identity indicates that H-atoms react similarly with the enzyme in its oxidized and reduced states as would be expected if initial actions were principally at sites in the protein. From the observed reduction efficiency of approx. 50 % it follows that half the H-atoms are captured at sites from which reducing equivalents cannot be transmitted to Fe(III). The resulting radicals are not manifest in the spectrum. Several types of radicals are known which may be among the products but would not be seen

in our spectra, for example, phenoxy, sulfur or amide nitrogen. The observation of several unresolvable intramolecular processes indicates the occurrence of parallel intramolecular reactions in which reducing equivalents are transmitted at different specific rates from different sites of initial action of H-atoms on different molecules of ferricytochrome *c*. This follows because spectral changes at all wavelengths measured proceed only in the direction of the spectrum of ferrocytochrome *c*. No spectral evidence was obtained for intermediates which may be formed in the transmission process but the absence of such evidence does not necessarily rule out transient existence of weakly absorbing intermediates.

It is clear from Figs 3 and 4 that a large fraction of the reduction of ferricytochrome *c* to the ferro form proceeds via an intramolecular process. In fact, the data of Figs 3 and 4 for $6 \cdot 10^{-5}$ M ferricytochrome *c*, taken together with $k$[H+ferricytochrome *c*] $= 1.0 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ show that when the bimolecular addition of H-atoms to ferricytochrome *c* is 95 % complete the reduction to ferrocytochrome *c* has achieved less than half of its final value. Similar data obtained with $8 \cdot 10^{-5}$ M ferricytochrome *c* show that at least 70 % of the spectral change due to the reduction results from intramolecular processes. This fraction, 70 %, can be regarded as a lower limit to the amount of reduction occurring via intramolecular transmission of reducing equivalents.

Free H-atoms are probably not identical with the form in which reducing equivalents are delivered to cytochrome *c* in the mitochondria. Furthermore, surface areas of the ferricytochrome *c* molecule in dilute aqueous solution may be available for acceptance of reducing equivalents which are not available in the more highly organized biological state; the multiplicity of intramolecular processes which we have observed may be a reflection of this wider availability. Our results do prove, however, that under the non-biological conditions of H-atom reaction, reduction equivalent transfer through the protein from initial peripheral sites of radical addition to the ferriheme moiety is demonstrable. It remains to be seen whether such processes occur under conditions which are more closely related to or identical with biological ones.

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