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REACTION OF CYTOCHROME *c* WITH ONE-ELECTRON REDOX REAGENTS

I. REDUCTION OF FERRICYTOCHROME *c* BY THE HYDRATED ELECTRON PRODUCED BY PULSE RADIOLYSIS*

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SUMMARY

Pulse radiolysis–kinetic spectrometry has been used to investigate the reaction of hydrated electrons with ferricytochrome *c* in dilute aqueous solution at pH 6.5–7.0. Time resolutions from $2 \cdot 10^{-7}$ to 1 s were employed. Transient spectra from 320 to 580 nm were characterized with a wavelength resolution of ± 0.5 nm. In neutral salt-free solution, k(ferricytochrome $c + e^-_{aq}$) $= (6.0 \pm 0.9) \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ and k(ferricytochrome $c + H$) $= (1.2 \pm 0.2) \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. The reaction of ferricytochrome *c* with hydrated electrons is sensitive to ionic strength; in 0.1 M $NaClO_4$, k(ferricytochrome $c + e^-_{aq}$) $= (2.4 \pm 0.4) \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. In contrast, k(ferricytochrome $c + H$) is insensitive to ionic strength. Time resolution of three spectral stages has been accomplished. The primary spectrum is the first observable spectrum detectable after irradiation and is formed in a second-order process. Its rate of formation is indistinguishable from the rate of disappearance of the electron spectrum. The secondary spectrum is generated in a true first order intramolecular process, $k(p \rightarrow s) = (1.2 \pm 0.1) \cdot 10^5$ s^{-1}. The tertiary spectrum is also generated in a true first-order process, $k(s \rightarrow t) = (1.3 \pm 0.2) \cdot 10^2$ s^{-1}. The specific rates of both transformations are independent of the wavelength of measurement. The tertiary spectrum, observable 50 ms after initial reaction and remaining unchanged thereafter for at least 1 s, shows that relaxed ferrocytochrome *c* is the only detectable product. This product is not autoxidizable, as expected for native reduced enzyme. It is more probable that the intramolecular changes responsible for the p→s and s→t spectral transformations involve the influence of conformational relaxation of ferrocytochrome *c* upon electronic energy states then that they are intramolecular transmission of reducing equivalents from primary sites of electron attachment.

INTRODUCTION

We have studied the reactions of simple one-electron equivalent redox reagents, the hydrated electron, e^-_{aq}, and atomic hydrogen, H, with ferricytochrome *c* in

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homogeneous aqueous solution near neutral pH. The action of ionizing radiation upon dilute solutions in water provides a convenient means of generating such one-electron redox reagents. Judicious choice of conditions allows individual redox species to be isolated and to react with otherwise unaffected substrates. The use of low concentrations of substrate, $0.25 \cdot 10^{-5}$–$5 \cdot 10^{-5}$ M in the present work, assures that direct action of ionizing radiation on the protein is negligible. Values of the yields of the reactive radiolytic species produced when water absorbs 100 eV of ionizing radiation assumed in this paper[2] are: H atom, 0.55; hydroxyl radicals, 2.74; and solvated electrons, 2.75. Other products of the radiolysis of water, H_2, H_2O_2 and H_3O^+, are not reactive under the conditions employed in the present work.

The reactive radicals, H, OH and e^-_{aq}, can be interconverted or selectively reacted to produce other radical species. Thus hydrated electrons can be converted into either H atoms or $\dot{O}H$ radicals:

$$e^-_{aq} + H^+ \rightarrow H \qquad k = 2.3 \cdot 10^{10}\,M^{-1} \cdot s^{-1} \qquad (1)^3$$

$$e^-_{aq} + H_2PO_4^- \rightarrow H + HPO_4^{2-} \qquad k = 1.5 \cdot 10^7\,M^{-1} \cdot s^{-1} \qquad (2)^3$$

$$e^-_{aq} + N_2O_{aq} \rightarrow N_2 + \dot{O}H + OH^- \qquad k = 5.6 \cdot 10^9\,M^{-1} \cdot s^{-1} \qquad (3)^3$$

Reactive hydroxyl radicals can be converted into unreactive 2,2-dimethyl-2-hydroxyethyl radicals by reaction with *tert*-butanol[4,5]:

$$\dot{O}H + (CH_3)_3COH \rightarrow H_2O + \dot{C}H_2C(CH_3)_2OH \qquad k = 3 \cdot 10^8\,M^{-1} \cdot s^{-1} \qquad (4)^3$$

The use of Reactions 1 and 4 allows isolation of H atoms as the only highly reactive species in acid solutions while the use of either 2 and 4 or of 3 and 4 provides isolated H atoms in neutral or alkaline solutions. There does not appear to be any widely useful method for removing H atoms selectively. However, since the radiolytic yield of H atoms is only about 20% of the yields of either $\dot{O}H$ or e^-_{aq} and its rate constant and mode of reaction with substrates can be separately established, allowance for its presence along with either of the latter can be made. Thus, Reaction 3 provides $\dot{O}H$ radicals in 10-fold excess over H atoms and Reaction 4 provides hydrated electrons in 5-fold excess over H atoms.

When the technique of pulse radiolysis is used, a radiation dose is delivered in a short time, in the present work, from 0.025 to 1.5 μs. Homogeneous distribution of the resulting radiolytic species is essentially achieved in approximately 1 ns. Thus, an absorbed dose of 100 rad provides a solution intitially $5.7 \cdot 10^{-8}$ M in H atoms, $2.8 \cdot 10^{-7}$ M in $\dot{O}H$ radicals and $2.8 \cdot 10^{-7}$ M in hydrated electrons. Kinetic absorption spectrometry can be used to follow the reactions of solutes with these species on time scales ranging from fractions of a μs to minutes. Pulse radiolysis–kinetic spectrometry has been used recently to study the dynamics of reactions of H, $\dot{O}H$ and e^-_{aq}, as well as other radicals produced by their selective action on cosolutes present in excess, with a number of proteins, *e.g.* lysozyme[6], ribonuclease[7–10], trypsin[11] and ferricytochrome *c*[1,12–15].

Early investigations[16–18] of reactions of ferricytochrome *c* with radiolytically produced redox reagents employed continuous radiation. A closely related study employed H atoms generated by the dissociation of molecular H_2 and introduced into the solution[19]. The earliest of these studies showed that irradiation of $1 \cdot 10^{-5}$ M

aqueous ferricytochrome *c* in the presence of 0.1 M succinate or benzoate produced ferrocytochrome *c*, as indicated by spectral data and reoxidation by cytochrome oxidase, while irradiation in the presence of 0.1 M ethanol produced denatured material[16]. Subsequent work[17] showed that irradiation in the presence of succinate, as well as β-hydroxybutyrate or glutamate, gave partial denaturation but that irradiation in the presence of malate or lactate gave ferrocytochrome *c* which appeared to have undergone little denaturation as indicated by spectral data and resistance of the product to autoxidation. These earliest studies were performed before the radiolysis of water was understood well enough to permit interpretation in terms of reactions of individual radicals. This understanding was first applied in a study[18] which, *inter alia*, suggested that radicals produced by the action of $\dot{O}H$ on the protein portion of a given molecule of ferricytochrome *c* reduce other molecules of the enzyme to its Fe(II) state.

Reaction with externally generated H atoms reduces ferricytochrome *c* to the ferro enzyme, as evidenced by the spectrum and resistance to autoxidation of the product; ferrocytochrome *c* is itself degraded by the further action of H atoms[19].

The study reported here employed the technique of pulse radiolysis–kinetic spectrometry to investigate the reaction of hydrated electrons with ferricytochrome *c* at pH 6.4–7.0. One of the objectives of this investigation was to use conditions of measurement sufficiently rigorous to minimize a variety of potential artifacts, *e.g.* the experimental difficulties inherent in applying the technique to a solute with an intense and finely structured absorption spectrum. Another was to characterize intramolecular transformations spectrally and kinetically so that chemical interpretation of these changes would be possible.

EXPERIMENTAL

Materials

Sigma Type VI horse heart ferricytochrome *c* (95–100% pure, prepared without use of trichloroacetic acid) was employed. The content of ferro enzyme, assayed *via* measurement of optical absorption at 550 nm[20] with a Cary 14 spectrophotometer was less than 7%. Solutions of the enzyme were prepared in water which had been distilled first from alkaline permanganate, then from acid dichromate and finally redistilled. Concentrations were determined routinely by weight but were calibrated by spectrophotometric determination of the concentration of the enzyme after its reduction by dithionite taking the molar extinction coefficient at 550 nm as $2.76 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (ref. 20). Solutions of the enzyme were prepared no more than 12 h before use and were stored in glass bottles. Their pH was adjusted to 6.4 to 7.0 by titration with $HClO_4$ or NaOH (Merck "Analyzed Reagent" and Baker "Analyzed Reagent", respectively). When desired, Fluka "Puriss. p.a." $NaClO_4$ was used to adjust ionic strength. N_2O was supplied by Matheson, 98% purity (remainder air). Argon was supplied by the Israel Oxygen Center. Both gases were routinely freed of oxygen by bubbling through alkaline aqueous pyrogallol (Baker and Adamson, "Purified Crystals"). Results obtained by this procedure were identical with those obtained by bubbling the gases through a solution containing V(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.

tert-Butanol (B.D.H. Reagent grade) contained no impurities detectable by ultraviolet absorption spectrometry.

Apparatus

The Varian linear accelerator at the Hebrew University was employed to produce 200-mA pulses of 5-MV electrons with pulse duration in the range 50–1500 ns. Some kinetic experiments employed 2.5-MV electrons (beam current, 1500 mA) in 25-ns pulses. A rectangular (1 cm×2 cm×2 cm) flow-through cell fabricated of high purity silica was employed. The optical path of the monitoring light was 2.0 cm. The light source was an Osram 150-W xenon lamp. Bausch and Lomb high-intensity monochromators (Models 33-86-01 and 33-86-02) were used in measurement of the primary and secondary spectra presented in Fig. 1 as well as of the kinetics of formation of the primary spectrum and its transformation into the secondary spectrum. Stray light was minimized with appropriate filters. A Hilger Watts double monochromator (Model D 330/331, 1200 groves·mm^{-1}) was used in measurement of the secondary and tertiary spectra presented in Figs 4–9 as well as of the kinetics of the secondary to tertiary transformation. Stray light was not detectable with this monochromator in the wavelength region of this work. Wavelength settings of both monochromators were calibrated routinely with interference filters (318, 544, 578–579 nm), the 467–468-nm line of the Osram 150-W xenon lamp, the 296.5-, 302.1-, 313.2-, 334.1- and 366.3-nm lines of an Hanovia 901 B-11 200-W Xe–Hg lamp and with the sharp absorption bands at 360, 417, 446–447, 453–454, 460 and 536 nm of a holmium glass filter. Slit widths used routinely with the Bausch and Lomb monochromators provided an optical band width of no more than ±1 nm as estimated by spectral resolution of the Soret band. Optical band widths provided by the Hilger double monochromator were estimated by spectral resolution of the holmium spectrum and Soret band to be no more than ±0.5 nm. Light transmitted by the monochromators was monitored by means of a 1 P 28 photomultiplier. Resulting voltages and times were measured with the aid of a Tektronix 556 double-beam oscilloscope or a Tektronix 549 storage oscilloscope.

Procedures

Deaeration of solutions and saturation with N_2O were accomplished by sweeping with argon or N_2O for at least 20 min. Solutions were deaerated and irradiated in large glass syringes equipped with capillary standard taper joints. Irradiations were carried out no more than 0.5 h after deaeration.

Absorbed dose per pulse was determined routinely using the spectrum of the solvated electron produced by pulsing $1 \cdot 10^{-2}$ M aqueous Matheson, Coleman and Bell Spectroquality ethanol at pH 9.5–10 taking $\varepsilon_{578} = 1.06 \cdot 10^4\ M^{-1} \cdot cm^{-2}$ and $G(e^-_{aq}) = 2.75$ molecules /100 eV.

All measurements were performed at 20±2 °C.

RESULTS

Spectra

The solutions employed in measuring spectra contained no added salts. The resulting spectra were therefore characteristic of the ionic strength provided by the

enzyme around neutral pH. A concentration of *tert*-butanol equal to 10^4 times the concentration of ferricytochrome *c* was present in all solutions. At least 98% of radiolytically generated hydroxyl radicals was removed by Reaction 4, thereby reducing available ȮH radicals to no more than 2% of the number of hydrated electrons.

The solutions were deaerated with argon. Only one pulse was delivered to a given aliquot of solution in determining spectra. Time resolution provided three spectral stages, the last of which was fully developed 50 ms after the pulse. No further change in spectrum was detectable up to 1 s after the pulse.

Fig. 1 compares spectra obtained with $2 \cdot 10^{-5}$ M enzyme 5 μs and 30 μs after a 1500-rad pulse (initial concentration of $e^-_{aq} + H = 5.1 \cdot 10^{-6}$ M), using the Bausch and Lomb high intensity monochromators, with a difference spectrum calculated from the data of Margoliash and Frohwirt[20]. In the calculation, it was assumed that each hydrated electron or H atom reduces one molecule of ferricytochrome *c* to the ferro form. Meaningful measurements could not be carried out for 400 nm $< \lambda <$ 420 nm under the conditions employed because of the high absorbance of the solution, $A > 3$. Under the conditions of Fig. 1, time resolution of the primary and secondary spectra is incomplete so that their differentiation is difficult. This follows because not only was generation of the primary spectrum (p) more than 98% complete 5 μs after the pulse but generation of the secondary spectrum (s) was almost 50% complete at

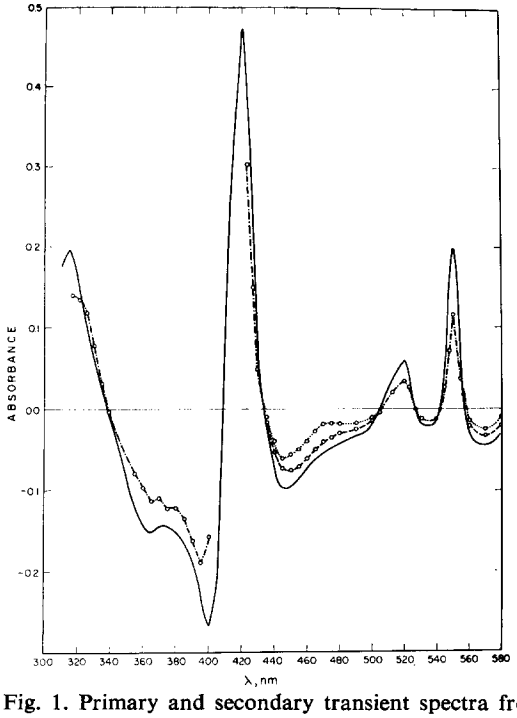

Fig. 1. Primary and secondary transient spectra from reaction of $2 \cdot 10^{-5}$ M ferricytochrome *c* at pH 6.8 with hydrated electrons + H atoms (Ar saturated): 1500 rad/pulse, initial $[e^-_{aq}]$ $4.2 \cdot 10^{-6}$ M, [H] $0.9 \cdot 10^{-6}$ M. O···O, 5 μs after pulse; O---O, 30 μs after pulse; O–·–O, 5-μs and 30-μs spectra indistinguishable; ———, spectrum calculated from data of ref. 20 assuming 100% efficient reduction by e^-_{aq}.

this time (see kinetics section). Thus, only larger p→s changes, as at 440–470 and 560–580 nm, could be detected. That a small change occurs in the α-band at 550 nm was established by applying 2000-rad pulses (initial $[H]+[e^-_{aq}]=6.8\cdot10^{-6}$ M) to $5\cdot10^{-5}$ M ferricytochrome *c*. Under these conditions, generation of the primary spectrum is more than 98% complete after 2 μs at which time the p→s process is no more than 20% complete. The p→s change at 550 nm consisted of a 5% increase in absorbance as shown in Fig. 2.

Spectral changes analogous to the p→s transformation of Fig. 1 were observed when ferricytochrome *c* was reacted with pulses of H atoms[12]. The kinetics of the H atom induced p→s change is similar to that of the p→s transformation induced under the conditions of the present work, where $([e^-_{aq}]/[H])=5$. The possibility that the spectral change depicted in Fig. 1 might originate in the action of H atoms or even be due to bimolecular reaction with $\dot{C}H_2C(CH_3)_2OH$ radicals was tested by employing conditions which provide only H atoms and $\dot{C}H_2C(CH_3)_2OH$ radicals. Thus, $2\cdot10^{-5}$ M ferricytochrome *c* containing 0.2 M *tert*-butanol and saturated with N_2O (approx. $1.5\cdot10^{-2}$ M) was subjected to 1500-rad pulses. About 99% of the electrons were thereby converted by Reaction 3 into $\dot{O}H$ radicals, at least 98% of which then reacted according to Reaction 4 to give $\dot{C}H_2C(CH_3)_2OH$ radicals. No more than 10% of H atoms is removed by this procedure since $k(H+$*tert*-butanol$)=10^{-5}\ k(H+$ferricytochrome *c*$)$[12,21]. These scavenging reactions supply the initial concentrations $[H]=8\cdot10^{-7}$ M and $[\dot{C}H_2C(CH_3)_2OH]=8.5\cdot10^{-6}$ M (taking primary $G_{OH}=2.75$ molecules/100 eV). This concentration of H atoms is the same as that which was produced under the conditions of Fig. 1 while the concentration of

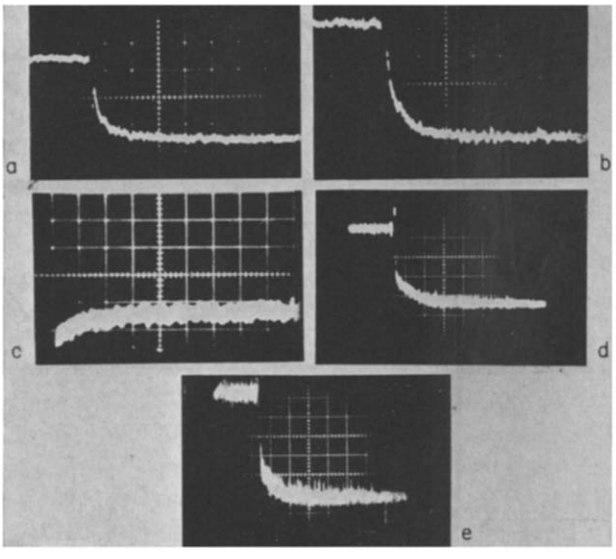

Fig. 2. Oscilloscope traces illustrating p→s spectral transformation initiated by electron attachment (Ar saturated). Abscissa scale 10 μs/division in all cases, ordinate scale (photomultiplier output voltage) variable. (a) $2\cdot10^{-5}$ M ferricytochrome *c*, 460 nm, 900 rad. (b) $2\cdot10^{-5}$ M, 460 nm, 1600 rad. (c) $5\cdot10^{-5}$ M, 550 nm, 2000 rad. (d) $5\cdot10^{-5}$ M, 580 nm, 3000 rad. (e) $5\cdot10^{-5}$ M, 580 nm, 1200 rad.

$\dot{C}H_2C(CH_3)_2OH$ radicals is twice as great. Very small spectral changes were observed under these conditions, where e^-_{aq} was replaced by *tert*-butanol radicals and where measurements were carried out with the same sensitivity as before. The primary and secondary spectra recorded in Fig. 1 can therefore be ascribed almost entirely to products of reaction with e^-_{aq}. Small but significant changes in absorbance were detected over a 50-μs period with measurements at higher sensitivity when N_2O-saturated *tert*-butanol solutions of ferricytochrome *c* were pulsed. These changes were confirmed by the larger signals obtained when more concentrated solutions containing the usual 10^4-fold excess of *tert*-butanol, were irradiated with 3000-rad pulses (Fig. 3a). Significant contributions to these very small spectral changes from bimolecular reaction of ferricytochrome *c* with $\dot{C}H_2C(CH_3)_2OH$ radicals can be ruled out on kinetic grounds (see section on s→t spectral changes). These small p→s spectral changes originate in the action of H atoms.

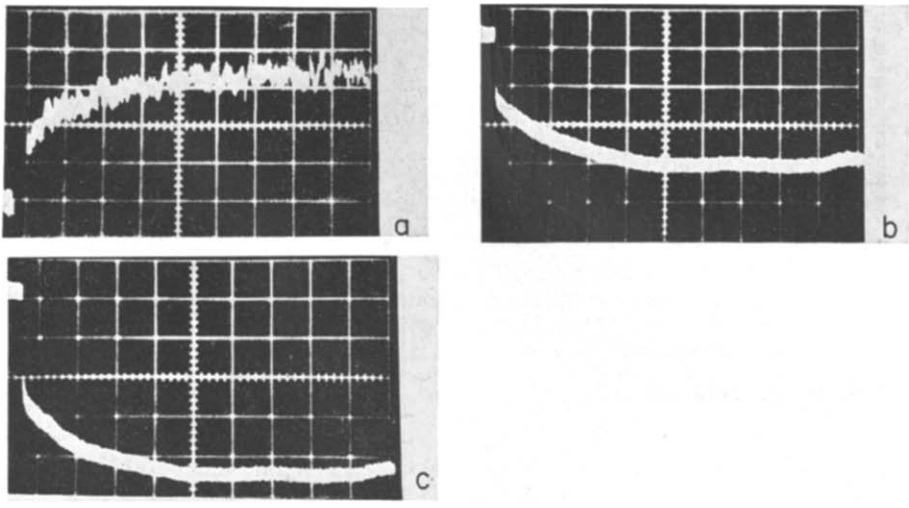

Fig. 3. Oscilloscope traces illustrating p→s and s→t spectral transformations initiated by reaction with H atoms (N_2O saturated). Abscissa scale as given, ordinate scale (photomultiplier output voltage) variable. (a) $1 \cdot 10^{-4}$ M ferricytochrome *c*, 550 nm, 3000 rad, 20 μs/division. (b) $1 \cdot 10^{-5}$ M, 460 nm, 900 rad, 2 ms/division. (c) $3 \cdot 10^{-5}$ M, 460 nm, 1500 rad, 2 ms/division.

Oscilloscope traces obtained under the conditions of Fig. 1 with sweep rates of the order of 2 ms·cm^{-1} showed small additional changes in absorbance. These changes were investigated using the Hilger double monochromator. In these experiments $1.0 \cdot 10^{-5}$ M ferricytochrome *c*, deaerated by sweeping with argon, was exposed to 500-rad pulses in the presence of 0.1 M *tert*-butanol. The resulting initial concentrations of hydrated electrons and H atoms were respectively $1.4 \cdot 10^{-6}$ M and $0.36 \cdot 10^{-6}$ M. Absorbances were measured with a precision of ± 0.0004. The secondary spectrum (s), measured 50 μs after the pulse, and the tertiary spectrum (t), measured 50 ms after the pulse are represented in Figs 4–9. The scales of these figures are expanded so that the relatively small differences between the secondary and tertiary spectra can be seen clearly. The shape of the spectrum calculated from the data of Margoliash and Frohwirt[20] is included for comparison.

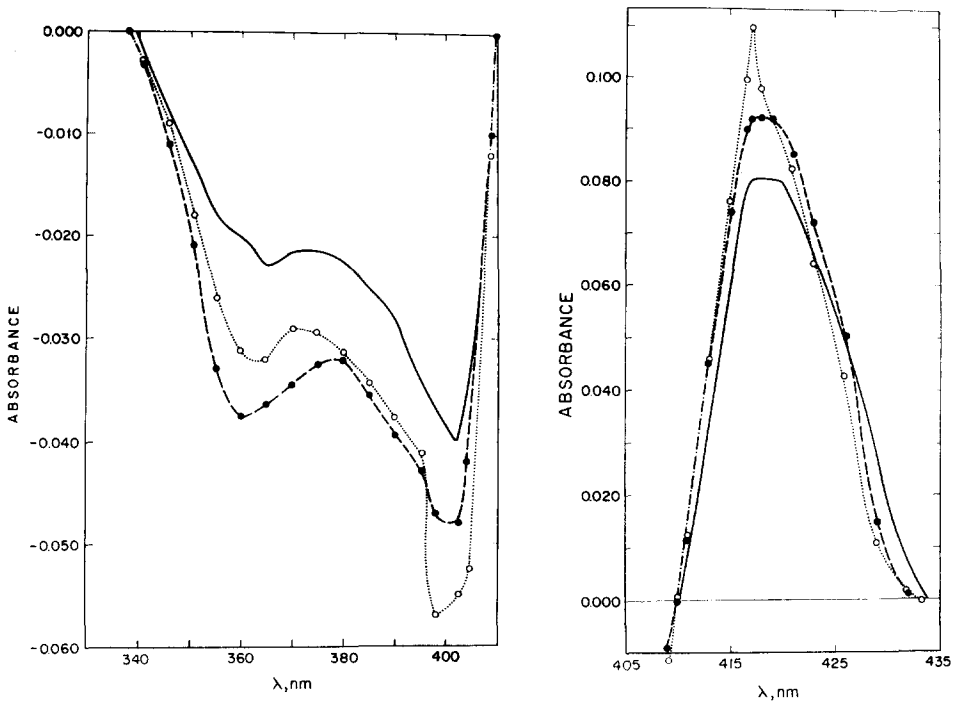

Fig. 4. Secondary and tertiary transient spectra from reaction at pH 6.6 of $1.0 \cdot 10^{-5}$ M ferricytochrome *c* with hydrated electrons + H atoms (Ar saturated): 500 rad/pulse, initial $[e^-_{aq}]$ $1.4 \cdot 10^{-6}$ M, [H] $0.3 \cdot 10^{-6}$ M. ○ · · · ○, 50 μs after pulse; ●---●, 50 ms after pulse; ———, calculated from data of ref. 20; 340–420 nm.

Fig. 5. Same as Fig. 4, 410–435 nm.

The possibility that the small s→t spectral changes might be due to bimolecular reaction of ferricytochrome *c* with $\dot{C}H_2C(CH_3)_2OH$ radicals is eliminated by the fact that the first-order specific rate of this transformation does not change significantly with 4-fold variation in the concentration of enzyme (Table I). The possibility remains that the s→t spectral change might be due at least in part to reaction with H atoms[12]. This possibility was again examined by comparing spectral data obtained with argon and N_2O swept solutions, all other conditions being maintained constant. Initial concentrations of H atoms were the same under the two conditions but the initial concentration of hydrated electrons was about 100 times as great in the argon-swept solution as with N_2O. The results are illustrated in Figs 3b and 3c and summarized in Table II. It is apparent that at 461, 553 and 580 nm reactions with hydrated electrons and with H atoms contribute comparable amounts to the s→t spectral transformation. Accordingly, H atoms appear 5 times more effective than electrons. In the Soret band (418 nm) on the other hand the electron reaction produces an 8-fold greater effect so that here e^-_{aq} appears nearly twice as effective as H atom.

The data of Figs 4–8 provide convincing evidence that the major species responsible for both secondary and tertiary spectra are ferricytochrome *c* and ferrocytochrome *c*. Table III compares the isosbestic wavelengths as well as wavelengths

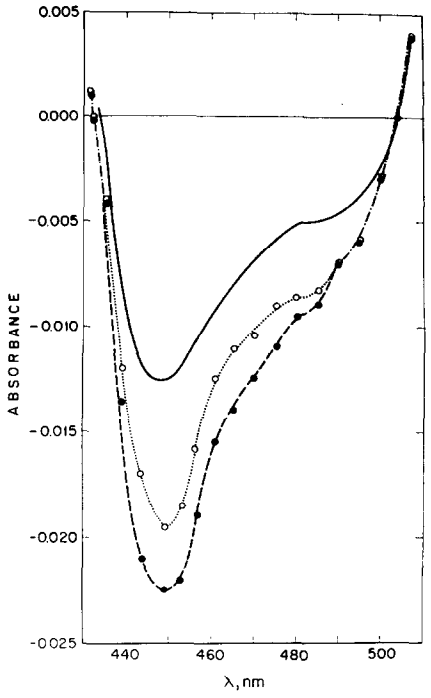

Fig. 6. Same as Fig. 4, 430–510 nm.

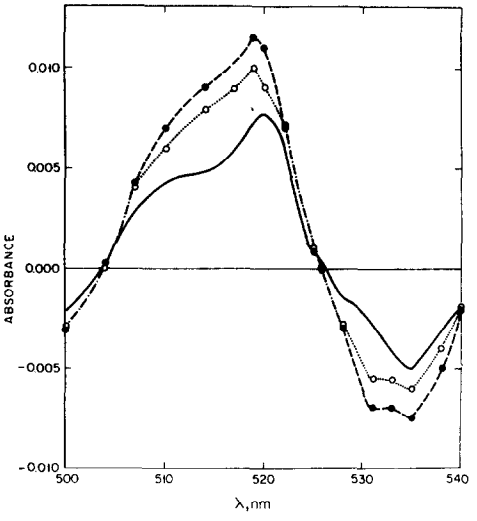

Fig. 7. Same as Fig. 4, 500–540 nm.

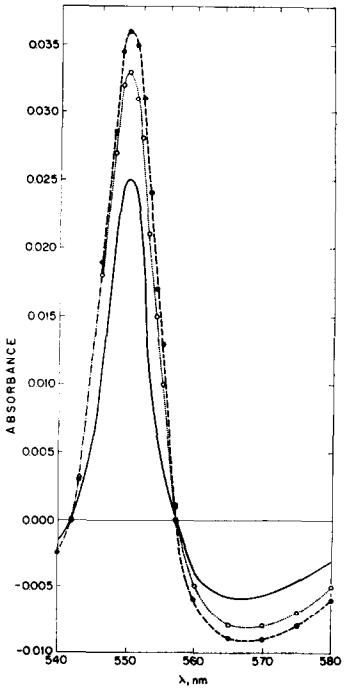

Fig. 8. Same as Fig. 4, 540–580 nm.

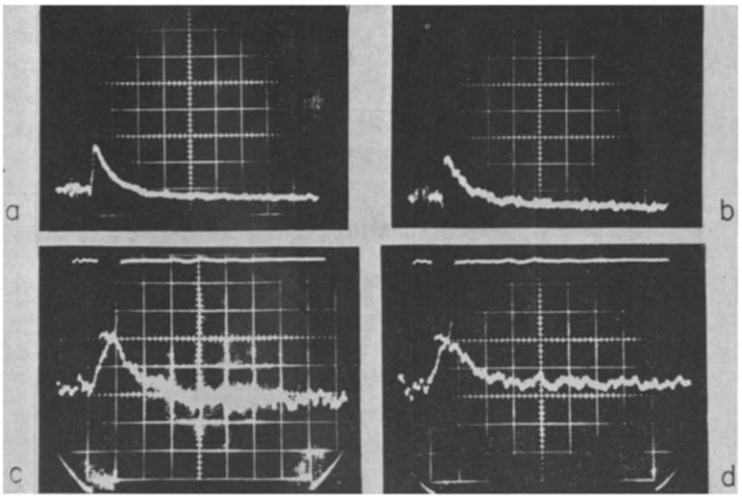

Fig. 9. Oscilloscope traces illustrating electron attachment to ferricytochrome *c* (Ar saturated). Abscissa scale as given, ordinate scale (photomultiplier output) variable. (a) $1 \cdot 10^{-5}$ M ferricytochrome *c*, 580 nm, 900 rad, 2 μs/division. (b) $1 \cdot 10^{-5}$ M, 580 nm, 400 rad, 2 μs/division. (c) $5 \cdot 10^{-5}$ M, 580 nm, 900 rad, 0.5 μs/division. (d) $5 \cdot 10^{-5}$ M, 650 nm, 900 rad, 0.5 μs/division.

TABLE I

FIRST-ORDER SPECIFIC RATES OF SECONDARY→TERTIARY SPECTRAL TRANSFORMATION AT pH 6.6

λ (*nm*)	[*Ferricytochrome c*] $\times 10^5$ (*M*)	*Dose/pulse* (*rad*)	$k(s \rightarrow t)^*$ $\times 10^{-2}$ (s^{-1})
370	4	3000	2.1 ± 0.3
417	1	300	1.1 ± 0.1
455	2	1500	1.1 ± 0.3
455	4	900	1.3 ± 0.3
455	4	1500	1.4 ± 0.2
455	4	1800	1.6 ± 0.1
455	4	3000	1.6 ± 0.1
550	2	1800	1.4 ± 0.2
550	4	1500	1.1 ± 0.2
550	4	3000	1.1 ± 0.2

* Uncertainties are mean deviations, measurements done in triplicate.

of minima and maxima from Figs 4–8 with values calculated from the data of Margoliash and Frohwirt[20]. The latter data were measured in 0.1 M phosphate buffer at pH 6.8 while the present data were obtained at almost the same pH in the absence of buffer. However, the spectra of unbuffered neutral solutions of ferricytochrome *c* and the product of its reduction by dithionite, measured on a Cary-14 spectrophotometer, were found to be virtually indistinguishable from the Margoliash–Frohwirt[20]

TABLE II

COMPARISON OF SECONDARY AND TERTIARY ABSORBANCES DUE TO REACTION WITH $\Sigma(e^-_{aq}+H)$ AND WITH H ATOMS ALONE

Secondary absorbances (A_s) measured 50 μs after the pulse. Tertiary absorbances (A_t) measured 50 ms after the pulse.

λ (*nm*)	$\Sigma(e^-_{aq}+H)$			*H atoms*		
	A_s	A_t	A_t-A_s	A_s	A_t	A_t-A_s
418*	0.100	0.092	−0.008	0.009	0.008	−0.001
461*	0.012	0.016	+0.004	0.001	0.003	+0.002
461**	0.062	0.078	+0.016	0.005	0.010	+0.005
553*	0.021	0.023	+0.002	0.002	0.003	+0.001
580*	0.005	0.006	+0.001	0.0005	0.001	+0.0005

* $1\cdot10^{-5}$ M ferricytochrome *c*, 0.1 M *tert*-butanol, 350 rad/pulse.
** $2.5\cdot10^{-5}$ M ferricytochrome *c*, 0.3 M *tert*-butanol, 1500 rad/pulse.

TABLE III

COMPARISON OF CHARACTERISTIC WAVELENGTH OF TERTIARY SPECTRUM WITH VALUES CALCULATED FROM DATA OF MARGOLIASH AND FROHWIRT[20]

$\lambda_{isosbestic}$ (*nm*)		λ_{max} (*nm*)		λ_{min} (*nm*)	
*Tertiary spectrum**	*Ref. 20***	*Tertiary spectrum**	*Ref. 20***	*Tertiary spectrum**	*Ref. 20***
338	339	317	315.5	355–370	355–370
410	410	417	417	398–402	400
433	434	519	520.5	445–450	445–450
504	504	549.5	550.25	535	535.25
525.5	526.5			560–570	560–570
542	541.75				
557.5	556.5				

* From data of Figs 4–9.
** Calculated from data of ref. 20 on the assumption that $A/[\text{ferrocytochrome } c]l=\varepsilon_{ferro}-\varepsilon_{ferri}$; see Eqn 8.

spectra. The close agreement between the spectral data of the present work and the values calculated from the spectra of authentic samples of oxidized and reduced cytochrome *c* is striking.

The product of reduction of ferricytochrome *c* by hydrated electrons resists autoxidation in a fashion similar to the behavior of native ferrocytochrome *c*[22]. The spectra in the α- and β-band region of a $5.4\cdot10^{-5}$-M neutral solution of ferricytochrome *c*, 0.5 M in *tert*-butanol and deaerated by argon sweeping, measured (against solvent blanks) under argon with a Cary-14 spectrophotometer a few minutes after exposure to a 1500-rad dose and again after sweeping with oxygen for 20 h,

were found to be identical. In contrast, 12 h of sweeping with oxygen oxidized a similarly treated solution which also contained 0.1 M $NaClO_4$ (pH 6.0) back to the ferri state.

TABLE IV

REDUCTION YIELDS FROM REACTION WITH e^-_{aq} AND WITH H ATOMS

λ (*nm*)	% *reduction yields* *	
	e^-_{aq}	*H*
418	68	30
461	73	60
553	78	46
580	75	57

* Calculated from A_t values of Table II and the spectral data of ref. 20 by means of the equation [ferrocytochrome c] $= A/l(\varepsilon_{\text{ferro}} - \varepsilon_{\text{ferri}})$.

Estimated reduction yields

The demonstration (Figs 4–8 and Table III) that the observed spectral changes are almost exactly those which accompany conversion of ferri- to ferrocytochrome *c* makes possible calculation of the efficiency of hydrated electrons in causing this transformation. Values given in Table IV are calculated from the tertiary spectrum and correspond to an efficiency of hydrated electrons of approximately 70%. The 10-fold excess of ferricytochrome *c* over hydrated electrons or $\dot{C}H_2C(CH_3)_2OH$ radicals and the very high value of k(ferricytochrome $c + e^-_{aq}$) (see below) exclude the possibility that more than 2 or 3% out of the total approx. 30% "inefficiency" could have been due to the trivial Reactions 5 and 6:

$$2e^-_{aq} \rightarrow H_2 + 2OH^- \qquad k = 5 \cdot 10^9\ M^{-1} \cdot s^{-1} \qquad (5)^3$$

$$e^-_{aq} + \dot{R} \rightarrow RH + OH^- \qquad k \approx 5 \cdot 10^9\ M^{-1} \cdot s^{-1} \qquad (6)$$

In the absence of a complete study of spectral changes caused by H atoms it can only be assumed that spectral changes caused by this reagent correspond to reduction of the enzyme. On this basis, the data of Table IV indicate an efficiency of H atoms of 30–60%.

Kinetics

Oscilloscope traces, unless otherwise indicated, were analyzed kinetically by transferring the data to punch cards by means of a magnifying manual trace follower coupled to an analog to digital converter and processing the cards in a Control Data Corp. 6400 digital computer.

Values of k(ferricytochrome $c + e^-_{aq}$) were calculated from traces, exemplified in Fig. 9, representing the fastest spectral changes which were observed under argon in the presence of a 10^4-fold excess of *tert*-butanol at neutral pH in the absence of added salts. The kinetic data for the individual traces corresponded well to first-order

behavior over the entire range of concentrations and wavelengths summarized in Table V. At given concentrations of ferricytochrome *c* the resulting pseudo-first-order rate constants were independent of dose per pulse over the range 350–1000 rad, *i.e.* upon reaction with initial concentrations of $1.0 \cdot 10^{-6}$–$2.8 \cdot 10^{-6}$ M of hydrated electrons and $0.20 \cdot 10^{-6}$–$0.57 \cdot 10^{-6}$ M of H atoms. Table V shows that second order rate constants calculated from the pseudo-first-order specific rates are independent of concentration of ferricytochrome *c* from $5 \cdot 10^{-6}$ to $5 \cdot 10^{-5}$ M. The substantially higher specific rates calculated for $2.5 \cdot 10^{-6}$ M enzyme presumably reflect the fact that the rate of consumption of hydrated electrons and H atoms by impurities is of the same order of magnitude as their consumption by ferricytochrome *c* at this low concentration. This explanation is supported by the observed rate of disappearance of the hydrated electron in similarly prepared solutions which lacked cytochrome *c*. The unweighted mean of the second-order specific rates, not including those calculated for $2.5 \cdot 10^{-6}$ M enzyme, and corresponding mean deviation are $(6.0 \pm 0.9) \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. A small number of measurements at 650 nm carried out at pH 6.1 with $1 \cdot 10^{-5}$ M enzyme and doses of 400 and 900 rad per pulse gave k(ferricytochrome $c + e^-_{aq}) = 8 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$. The initial concentration of H atoms and their specific rate[12] of reaction with ferricytochrome *c* are much lower than the corresponding values for hydrated electrons. In addition, the efficiency of reduction by H atoms appears to be only about two-thirds that by hydrated electrons. The contribution of the reaction with H atoms to the measured value of k(ferrichtochrome $c + e^-_{aq}$) is therefore negligible.

The effect of ionic strength on k(ferricytochrome $c + e^-_{aq}$) at pH 6.0–6.1 was evaluated by measurements carried out with $1 \cdot 10^{-5}$ and $2 \cdot 10^{-5}$ M ferricytochrome *c*, 0.1 M in $NaClO_4$, 0.1 or 0.2 M, respectively, in *tert*-butanol and deaerated by sweeping with argon. The rate constant determined at 370, 580 and 650 nm was $(2.4 \pm 0.4) \cdot 10^{10}$ M^{-1} s^{-1}, independent of dose per pulse from 350 to 1500 rad per

TABLE V

PSEUDO-FIRST-ORDER AND SECOND-ORDER SPECIFIC RATES OF e^-_{aq} REACTION WITH FERRICYTOCHROME *c* AT pH 6.8–7.2

Dose per pulse varied from 350 to 1000 rad. Uncertainties are mean deviations; where indicated at least five measurements performed.

λ (*nm*)		[*Ferricytochrome c*] $\times 10^5$ (*M*)			
		0.25	*0.5*	*1*	*5*
510	$k_1 \times 10^{-6}$ (s^{-1})	—	—	0.53 ± 0.10	—
	$k_2 \times 10^{-10}$ ($M^{-1} \cdot s^{-1}$)	—	—	5.3 ± 1.0	—
520	$k_1 \times 10^{-6}$ (s^{-1})	0.23 ± 0.3	—	0.53 ± 0.14	3.0
	$k_2 \times 10^{-10}$ ($M^{-1} \cdot s^{-1}$)	9.0 ± 1.2	—	5.3 ± 1.4	6.0
535	$k_1 \times 10^{-6}$ (s^{-1})	0.22 ± 0.03	—	0.68 ± 0.17	3.0
	$k_2 \times 10^{-10}$ ($M^{-1} \cdot s^{-1}$)	9.0 ± 1.2	—	6.8 ± 1.7	6.0
580	$k_1 \times 10^{-6}$ (s^{-1})	0.25 ± 0.08	0.30 ± 0.07	0.62 ± 0.07	3.0 ± 0.3
	$k_2 \times 10^{-10}$ ($M^{-1} \cdot s^{-1}$)	10 ± 3	6.0 ± 1.4	6.2 ± 0.7	6.0 ± 0.6
650	$k_1 \times 10^{-6}$ (s^{-1})	0.24 ± 0.04	0.28 ± 0.04	0.62 ± 0.08	3.0 ± 0.3
	$k_2 \times 10^{-10}$ ($M^{-1} \cdot s^{-1}$)	10 ± 2	5.6 ± 0.8	6.2 ± 0.8	6.0 ± 0.6

pulse. At pH 7.1 with $1 \cdot 10^{-5}$ M ferricytochrome *c*, 0.1 M in $NaClO_4$, *k*(ferricytochrome $c + e^-_{aq}$) was found to be $(2.0 \pm 0.2) \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ independent of wavelength and dose per pulse.

Values of *k*(ferricytochrome c + H) at neutral pH were calculated in a similar fashion from traces produced at 460 and 550 nm with N_2O-saturated solutions of ferricytochrome *c* containing 10^4-fold excesses of *tert*-butanol. The second-order specific rate, independent of concentration of ferricytochrome *c* ($2 \cdot 10^{-5}$ and $3 \cdot 10^{-5}$ M) and wavelength, was $(1.2 \pm 0.2) \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$.

The kinetics of the p→s spectral change occurring in argon-deaerated, neutral, salt-free solutions containing a 10^4-fold excess of *tert*-butanol was measured by analysis of oscilloscope traces exemplified in Fig. 2. As shown above, under the conditions employed, the p→s spectral change is almost entirely a consequence of action by hydrated electrons. Individual traces agreed well with first-order kinetics. The first-order specific rates did not vary with 2.5-fold variation of concentration of ferricytochrome *c* or with 5-fold variation of dose per pulse. The data summarized in Table VI establish that, at the wavelengths measured, the p→s spectral transformation elicited by the action of hydrated electrons on ferricytochrome *c* is truly first order. The value of $k_{e^-_{eq}}$(p→s) is $(1.2 \pm 0.1) \cdot 10^5$ s^{-1}, independent of wavelength. In the presence of 0.1 M $NaClO_4$, with $5 \cdot 10^{-5}$ M enzyme at pH 7.2, spectral changes occurring after initial addition of the electron were studied at 460 and 580 nm. The magnitudes of changes in absorbance were similar to those observed in the absence of added salt but time resolution of separate stages was not possible at time scales from 10 $\mu s \cdot cm^{-1}$ to 1 $ms \cdot cm^{-1}$.

The kinetics of the p→s spectral transformation associated with the action of H atoms was measured in neutral, salt-free, N_2O-saturated $1 \cdot 10^{-4}$ M ferricytochrome *c*, containing 1 M *tert*-butanol, upon exposure to doses of 2200 and 3000 rad per pulse. Individual traces agreed well with first-order kinetics. Dependence on concentration of enzyme was not investigated. Measurements at 550 and 470 nm gave k_H(p→s) =

TABLE VI

FIRST-ORDER SPECIFIC RATES OF PRIMARY→SECONDARY SPECTRAL TRANSFORMATION

λ (nm)	$k(p \rightarrow s) \times 10^{-5}$ (s^{-1}) [Ferricytochrome *c*] $\times 10^5$ (*M*):								
	2			*3*			*5*		
	Dose/pulse (rad):								
	600	*1000*	*3000*	*600*	*1000*	*3000*	*600*	*1000*	*3000*
370	—	—	1.3*	—	—	—	—	—	—
450	1.3	1.3	1.2	—	—	—	1.3	1.3	1.3
480	1.4	1.3	1.4	—	—	—	—	—	—
505	—	1.3	1.3	—	1.3	1.2	—	1.3	1.2
550	—	—	—	—	—	—	—	—	1.4*
580	1.2	1.2	1.1	1.0	1.2	1.1	1.0	1.0	1.1

* 2000 rad/pulse.

$(3.8 \pm 0.3) \cdot 10^4$ s^{-1}, measured by method of half lives for three half lives, independent of wavelength and dose per pulse.

As shown in Table II and discussed above, it was not possible to isolate the s→t spectral transformation caused only by hydrated electrons. Transformations, exemplified in Fig. 10, elicited significantly by both hydrated electrons and H atoms contribute when neutral, salt-free, argon-purged solutions of enzyme are irradiated in the presence of a 10^4-fold excess of *tert*-butanol. The data of Table I, show that the s→t transformation is truly first order, independent of concentration of ferricytochrome *c* over a 2-fold range and of dose per pulse over a 3-fold range. The value of $k_{e^-_{aq}+H}{}^*$ (s→t) in the 417–450-nm range, measured by the method of half lives for three half lives, is $(1.3 \pm 0.2) \cdot 10^2$ s^{-1} apparently independent of wavelength.

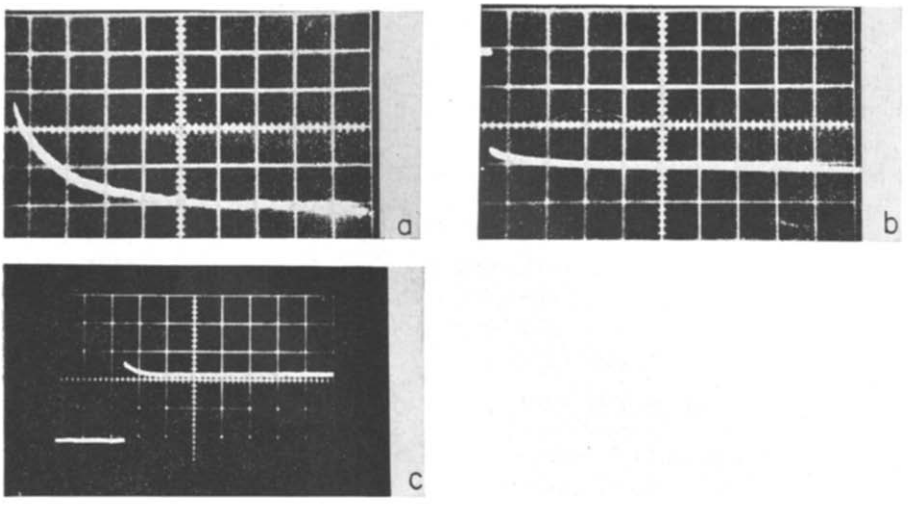

Fig. 10. Oscilloscope traces illustrating s→t spectral transformation initiated by $e^-_{aq}+H$ (Ar saturated), ordinate scale (photomultiplier output voltage) variable. (a) $3 \cdot 10^{-5}$ M ferricytochrome *c*, 460 nm, 1500 rad, 2 ms/division. (b) Same as a, except 10 ms/division. (c) $1 \cdot 10^{-5}$ M ferricytochrome *c*, 414 nm, 300 rad, 10 ms/division.

The kinetics of the s→t spectral transformation originating solely in the action of H atoms was measured using N_2O-saturated, salt-free solutions containing a 10^4-fold excess of *tert*-butanol. The method of half lives was employed for three half lives. At 460 nm, for $1 \cdot 10^{-5}$ and $2.7 \cdot 10^{-5}$ M ferricytochrome *c* and doses per pulse of 1500 and 2000 rad, k_H (s→t) $= (2.8 \pm 0.2) \cdot 10^2$ s^{-1}. At 550 nm for $1 \cdot 10^{-5}$ M enzyme and doses per pulse of 1500 and 2000 rad, k_H (s→t) $= 1.3 \cdot 10^2$ s^{-1}.

DISCUSSION

Comparison with prior work

Pulse radiolysis–kinetic spectrometry has been used by Pecht and Faraggi[14] to investigate the reaction of hydrated electrons with horse heart ferricytochrome *c* in neutral, salt-free solution. They describe a primary spectrum which is significantly shifted towards the blue in the Soret band and state that this transforms into the nor-

mal spectrum of ferrocytochrome *c* with a first-order specific rate of $8.5 \pm 0.3\ s^{-1}$. Neither the blue shift of the Soret band nor a spectral transformation with a first-order specific rate near $8.5\ s^{-1}$ was observed in the present work. Pecht and Faraggi[14] apparently did not observe the p→s ($1.2 \cdot 10^5\ s^{-1}$) or s→t ($1.3 \cdot 10^2\ s^{-1}$) spectral transformations which were observed in the present work. The possibility that their measurements in the Soret band were not completely accurate is at least suggested by the fact the conditions of measurement ($1 \cdot 10^{-5}$ M enzyme and a light path of 12.4 cm) which they describe correspond to an absorbance of more than 10 in the Soret band. Reliable measurement of relatively small changes of absorbance, *i.e.* up to 0.1, as they report for the Soret band, is very difficult under such circumstances.

Pecht and Faraggi[14,23] also report a value of *k*(ferricytochrome $c + e^-_{aq}$) up to 3 times as large as the value found in the present work. They employed very low concentrations of enzyme in their work, *i.e.* $0.5 \cdot 10^{-6}$ to $4 \cdot 10^{-6}$ M, and their results show a smooth decrease of the second order specific rate with increasing concentration. The data of the present work, as discussed above, suggest that measurements at such low concentration may be complicated by the presence of adventitious impurities which react with hydrated electrons. The value of *k*(ferricytochrome $c + e^-_{aq}$) found in the present work is in better agreement with the value reported by Lichtin *et al.*[12] $4 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$, for the reaction of horse heart ferricytochrome *c* in salt-free solution.

Land and Swallow[13] employed pulse radiolysis–kinetic spectrometry to investigate the reaction of hydrated electrons with ferricytochrome *c* in the presence of 0.1 M sodium formate. Formate ion scavenges hydroxyl radical to give the carboxylate radical, as shown in Eqn 7:

$$\dot{O}H + HCO_2^- \rightarrow H_2O + CO_2^{\dot{-}} \qquad k = 2.5 \cdot 10^9\ M^{-1} \cdot s^{-1} \tag{7}$$

The carboxylate radical, $CO_2^{\dot{-}}$, is itself a reducing agent which reacts more slowly[13] with ferricytochrome *c*, *k*(ferricytochrome $c + CO_2^{\dot{-}}$) $\approx 5 \cdot 10^8\ M^{-1} \cdot s^{-1}$, a reaction which would obscure first-order spectral changes with specific rates of the order of 10^3–$10^4\ s^{-1}$. These authors do not report first-order spectral transformations with specific rates comparable to *k*(p→s) or *k*(s→t). As noted in the Results section, resolution of such stages was not possible in the presence of 0.1 M $NaClO_4$. Land and Swallow[13] observed a slow first-order spectral transformation in the Soret band, specific rate = $7.3\ s^{-1}$, at pH 8.3 and 9.2 but not at pH 6.8. Direct comparison of the spectral data of Land and Swallow with those of Pecht and Faraggi or the present work is complicated by the possibly dominant influence of ionic effects[22]. However, data are avaialable to make possible comparison of *k*(ferricytochrome $c + e^-_{aq}$). Land and Swallow report a value of $2 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ in the pH range 6–7 (0.1 M $NaHCO_2$ *plus* 0.01 M phosphate buffer), in good agreement with the values found in the present work when 0.1 M $NaClO_4$ was present, $(2.4 \pm 0.4) \cdot 10^{10}$ and $(2.0 \pm 0.2) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ at pH 6 and 7, respectively. The substantial negative salt effect on *k*(ferricytochrome $c + e^-_{aq}$) observed in the present work is not surprising in view of the high positive charge on ferricytochrome *c* in neutral solution[22]. In contrast, *k*(ferricytochrome *c* + H) is insensitive to both pH and ionic strength. Its value found in the present work for neutral salt-free solutions, $(1.2 \pm 0.2) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$, agrees well with the value previously reported[12] for salt-free solutions at pH 2.5 or in 0.3 M phosphate buffer at pH 6.8, $1 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. It is to be expected that the specific

rate of reaction of uncharged H atoms should be essentially independent of Debye–Hückel type salt effects. The magnitude of k(ferricytochrome c+H) is apparently also not very sensitive to changes in the tertiary structure of the protein associated with differences in pH or salt concentration.

Land and Swallow were unable to obtain evidence of fast spectral changes subsequent to electron addition which might indicate fast internal transmission of redox equivalents[13]. From their experimental conditions they concluded that the specific rate of any such process must be greater than $4 \cdot 10^6$ s^{-1}.

Analysis of results

The present study establishes small but unequivocal first-order intramolecular spectral changes subsequent to electron addition. Since neither buffer nor inert salt was present, these changes must be due to intramolecular processes within the enzyme molecule, possibly but not necessarily involving water. The data do not allow a firm conclusion to be drawn as to whether the p→s and s→t spectral changes are associated with consecutive or parallel molecular processes.

The possibility exists that the transmission of reducing equivalents from sites of initial capture of the electron to heme iron and/or conformational relaxation associated with the change in the oxidation state of iron[24,25] are responsible for the p→s and s→t spectral changes. In order to examine the possible significance of the differences between the p, s and t spectra it is necessary to recognize that the absorbances plotted in Figs 1 and 4–8 are based on measurements in which the "blank" is solution prior to pulsing, *i.e.* ferricytochrome *c*, and the "sample" is solution after pulsing and contains reacted solute along with several times as much unreacted ferricytochrome *c*. For any single absorbing species X_i, formed in concentration $[X_i]$ from an initial species X_0, the measured absorbance at a given wavelength is given by Eqn 8 where A/l is the absorbance per unit path length and ε_0, ε_i are the molar absorbance indices of the absorbing species:

$$A/l=(\varepsilon_i-\varepsilon_0)\,[X_i] \tag{8}$$

The calculated spectra in Figs 1 and 4–8 identify ε_i and ε_0, respectively, with the molar absorbance indices[20] of equilibrated ferrocytochrome *c* and ferricytochrome *c*. For the experimental primary, secondary and tertiary transient spectra ε_0 is also the molar absorbance index of ferricytochrome *c*. Table III and Figs 4–8 show that the tertiary spectrum is very similar to a spectrum calculated for a mixture of ferricytochrome *c* with equilibrated ferrocytochrome *c* and it can be assumed that contributions from other absorbing species are negligible 50 ms after the pulse. A striking feature of both the p→s and s→t spectral transformations is the essentially negligible dependence of their specific rates on wavelength. This indicates that p→s is due to a single molecular transformation (or set of similar transformations) while s→t is due to another such event (or set of events). Even if parallel molecular processes are involved, it is not possible to tell whether the two events occur in the same molecule or in different molecules.

The most prominent differences between the tertiary and secondary spectra are differences in intensity at maxima and minima, summarized in Table VII. Aside from a small but significant distortion of the secondary spectrum in the Soret band region, differences in the wavelengths of maxima and minima and isosbestic points

TABLE VII

RELATIVE VALUES OF $A_t - A_s$ AT MAXIMA AND MINIMA

λ (*nm*)	*Spectral Feature*	$\frac{A_t - A_s}{A_t}$
360	min	−0.17
398	min	+0.21
417	max	−0.20
449	min	−0.13
519	max	+0.13
535	min	−0.21
550	max	+0.08
567	min	−0.11

are negligible. If the s→t spectral change reflects transmission of a reducing equivalent from a single type of radical site to heme iron in a fraction of the enzyme molecules which underwent electron attachment, then the difference between the s and t spectra can be expressed by Eqn 9, where ε_{ferro} refers to equilibrated ferrocytochrome *c* and $\dot{R}$ refers to the radical site:

$$\frac{A_t - A_s}{l} = (\varepsilon_{ferro} - \varepsilon_{\dot{R}}) [\dot{R}] \tag{9}$$

This model requires that $\dot{R}$, ferro- and ferricytochrome *c* share common isosbestic points. It also requires that $\varepsilon_{\dot{R}}$ at 417 nm be greater than $1.28 \cdot 10^5$, the value for ε_{ferro}[20]. Although it is possible that these requirements can be met, it is not probable. In contrast, much larger differences in values of ε than in the wavelengths of maxima, minima and isosbestic points are to be expected if the s→t spectral transformation is due to conformational relaxation. The spectra of natural and synthetic ferrohemochromes display much larger relative sensitivity of intensities to the environment of the heme than of characteristic wavelengths[26]. Analysis of the p→s spectral transformation is handicapped by the less complete and less accurate data. Similar arguments, however, suggest that molecular relaxation is the more probable source of this change.

The tertiary spectrum corresponds to about 70% efficient use of hydrated electrons in the conversion of ferro- to ferricytochrome *c*. It can be shown that little of the approx. 30% deficiency from 1:1 stoichiometry can be due to reactions of electrons with each other or with radicals derived from *tert*-butanol. Presumably about 30% of the electrons attach to the enzyme at sites which do not give reduction of heme iron. The spectral data indicate that the resulting radical sites have little effect on the spectrum of the ferricytochrome *c* molecules in which they are located.

While we ascribe the first-order intramolecular changes initiated by the addition of e^-_{aq} to ferricytochrome *c* largely to conformational changes, we wish to point out that such changes are necessarily associated with modification of electron distribution in the reduced enzyme. As a result of both conformational and associated electronic changes the ability of resultant ferrocytochrome *c* to transfer an electron to acceptors, *in vitro* or *in vivo*, may alter in the time scale of the observed trans-

formations, *i.e.* $1 \cdot 10^{-6}$ to $1 \cdot 10^{-2}$ s. The intramolecular changes resulting on the addition of H atoms[12] are different from those initiated by the action of e^-_{aq}. It is not, as yet, possible to assess the relative contributions of conformational relaxation and intramolecular transmission of reducing equivalents consequent upon addition of H atoms.

Whether intramolecular transmission of reducing equivalents consequent upon addition of hydrated electrons to ferricytochrome *c* occurs with a specific rate greater than the limit of detection in these experiments, approx. $3 \cdot 10^5$ s^{-1}, remains an open question.

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REFERENCES

1 Lichtin, N. N., Shafferman, A. and Stein, G. (1973) *Science* 179, 680–682
2 Draganić, I. G. and Draganić, Z. D. (1971) *The Radiation Chemistry of Water*, pp. 47–107, 123–168, Academic Press, New York
3 Anbar, M. and Neta, P. (1967) *Int. J. Appl. Radiat. Isot.* 18, 493–523
4 Scholes, G. and Simic, M. (1964) *J. Phys. Chem.* 68, 1738–1743
5 Simic, M., Neta, P. and Hayon, E. (1969) *J. Phys. Chem.* 73, 3794–3800
6 Adams, G. E., Willson, R. L., Aldrich, J. E. and Cundall, R. B. (1969) *Int. J. Radiat. Biol.* 16, 333–342
7 Adams, G. E., Willson, R. L., Bisby, R. H. and Cundall, R. B. (1971) *Int. J. Radiat. Biol.* 20, 405–415
8 Adams, G. E., Bisby, R H., Cundall, R. B., Redpath, J. L. and Willson, R. L. (1972) *Radiat. Res.* 49, 290–299
9 Lichtin, N. N., Ogdan, J. and Stein, G. (1972) *Biochim. Biophys. Acta* 263, 14–30
10 Lichtin, N. N., Ogdan, J. and Stein, G. (1972) *Biochim. Biophys. Acta* 276, 124–142
11 Masuda, T., Ovadia, J. and Grossweiner, L. J. (1971) *Int. J. Radiat. Biol.* 20, 447–459
12 Lichtin, N. N., Ogdan, J. and Stein, G. (1971) *Israel J. Chem.* 9, 579–582
13 Land, E. J. and Swallow, A. J. (1971) *Arch. Biochem. Biophys.* 145, 365–372
14 Pecht, I. and Faraggi, M. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 902–906
15 Nilsson, K. (1972) *Israel J. Chem.* 10, 1011–1019.
16 Mee, L. K. and Stein, G. (1956) *Biochem. J.* 62, 377–380
17 Munk, S. and Stein, G. (1958) *Proc. 2nd Int. Conf. Peaceful Uses At. Energy* 22, 535–536
18 Rabani, J. and Stein, G. (1962) *Radiat. Res.* 17, 327–340
19 Czapski, G., Frohwirt, N. and Stein, G. (1965) *Nature* 207, 1191–1192
20 Margoliash, E. and Frohwirt, N. (1959) *Biochem. J.* 71, 570–571
21 Neta, P., Fessenden, R. W. and Schuler, R. H. (1971) *J. Phys. Chem.* 75, 1654–1666
22 Margoliash, E. and Schejter, A. (1966) *Adv. Protein Chem.* 21, 113–286
23 Pecht, I. and Faraggi, M. (1971) *FEBS Lett.* 13, 221–224
24 Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. and Margoliash, E. (1971) *J. Biol. Chem.* 246, 1511–1533
25 Takano, T., Swanson, R., Kallai, O. B. and Dickerson, R. E. (1971) *Cold Spring Harbor Symp. Quant. Biol.* 36, 397–404
26 Blauer, G. and Yonath, A. (1967) *Arch. Biochem. Biophys.* 121, 587–595