Biochimica et Biophysica Acta, 368 (1974) 86–96

BBA 46811

ONE-ELECTRON REACTIONS IN BIOCHEMICAL SYSTEMS AS STUDIED BY PULSE RADIOLYSIS

VI. STAGES IN THE REDUCTION OF FERRICYTOCHROME *c*

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(Received May 1st, 1974)

SUMMARY

When ferricytochrome *c* at pH about 9 is reduced by hydrated electrons and/or CO_2^-, it gives rise to an unstable form of ferrocytochrome *c* whose absorption spectrum, particularly in the Soret region, differs from that of normal ferrocytochrome *c*. This form changes intramolecularly (life-time about 0.1 s at ambient temperature) to yield normal ferrocytochrome *c*, and by 0.5 s the change in absorption spectrum in the range 225–600 nm produced by e^-_{aq} and/or CO_2^- is identical to the final change produced by reduction with an equivalent amount of sodium dithionite. This shows that both e^-_{aq} and CO_2^- reduce cytochrome *c* with practically 100 % efficiency. In the range 600–800 nm the spectrum of the unstable form is the same as that of normal ferrocytochrome *c*, both having small absorptions at 695 nm as compared with ferricytochrome *c*. As the unstable form disappears however a further loss of absorption at 695 nm occurs. This is taken to imply that the unstable form decays to a second unstable form which then rapidly donates an electron to the unchanged neutral form of ferricytochrome *c*, so reducing absorption in the 695 nm band. Subsequent to this process the absorption in the 695 nm band increases over a period of minutes owing to re-equilibration between the neutral and alkaline formes of ferricytochrome *c*. Between pH 7 and 10 the effect of pH on the absorption changes is consistent with the hypothesis of a second unstable form of ferrocytochrome *c*. Additional phenomena arise in more alkaline solutions. The rates of the various unimolecular processes are thought to be determined by the rates of change of conformation of the protein parts of the molecule following the change in oxidation state.

INTRODUCTION

Pulse radiolysis experiments have shown that hydrated electrons react rapidly with cytochrome *c* in its oxidized form [1–3] in part by direct interaction with the haem moiety and in part via other parts of the molecule. There is evidence that Tyrosine 67 plays a role in the reaction [4]. Hydrogen atoms [5] and free CO_2^- [1] and other organic [6] radicals also cause rapid reduction. The rapidity of all these reductions

has permitted direct observation of subsequent intramolecular changes, and processes with first-order rate constants varying from 1.7 s^{-1} [2] to 10^5 s^{-1} [7, 8] have been reported, and attributed to internal transfer of reducing equivalents or to conformational changes. Further observations have now been made, mainly on mildly alkaline solutions, with special emphasis on changes occurring at the 695 nm absorption band. The results provide new information about intermediate stages in the reduction process.

METHODS

Horse heart cytochrome *c* was obtained from Koch-Light (90–100 %) or Sigma (Type VI). Solutions of known concentration were made up by weighing the material, taking the water content into account. The absorption of the solutions at 695 nm was checked before the experiments, and direct comparison showed no difference between cytochrome *c* from the two sources in the rate or extent of changes in absorbance produced by giving pulses of radiation to deaerated solutions which also contained sodium formate at pH 9.2. Water was distilled from alkaline permanganate. Other chemicals were of Analar quality. Solutions were either deaerated by bubbling with argon (Air Products) or saturated with N_2O (B.O.C.).

Pulse radiolysis experiments were conducted as in earlier studies in this series [1], using single pulses (< 1 μs duration) of electrons (approx. 10 MeV) from a Vickers linear accelerator. Solutions were contained in cells of optical path length 1 mm (Figs. 1 and 4, Tables I and II) 2.5 cm (Fig. 2) or 5 cm (Fig. 3). A single Bausch and Lomb monochromator was used in most of the experiments. The bandwidth was normally 2.5 nm. The accuracy of the wavelength scale was checked using the known absorption bands of cytochrome *c* or interference filters.

Absorption spectra of stable solutions were determined with a Uvispek H700 (Hilger), a Spectronic 505 (Bausch and Lomb) or a digital ultraviolet spectrophotometer (Cecil Instruments).

RESULTS

Previous experiments have indicated that in mildly alkaline solutions, hydrated electrons and CO_2^- appear to react with cytochrome *c* to form a product whose absorption in the Soret region differs from that of normal reduced cytochrome *c* [1]. The product then seemed to disappear slowly (with a rate constant at 19 °C of 7.3 s^{-1}, activation energy 85 kJ/mole) apparently to form normal reduced cytochrome *c*. To establish the stoichiometry of the reduction, careful measurements have now been made in the wavelength range 225–600 nm of the difference between the absorbance of cytochrome *c* solutions before the pulse and the absorbance (a) after hydrated electrons and CO_2^- have reacted but before the onset of the subsequent slow process (about 1 ms) and (b) after completion of the slow process (0.5 s). The solutions contained $9.3 \cdot 10^{-5}$ M cytochrome *c*, of which $8.4 \cdot 10^{-5}$ M was in the oxidized form, together with 0.1 M sodium formate, and were buffered to pH 9.2 with 10^{-2} M borate buffer. Air was removed from the solutions by bubbling with argon. Each pulse delivered $3.2 \cdot 10^3$ rad so that most of the hydrated electrons and CO_2^- would react with the cytochrome *c* rather than with each other. From the rate constants for

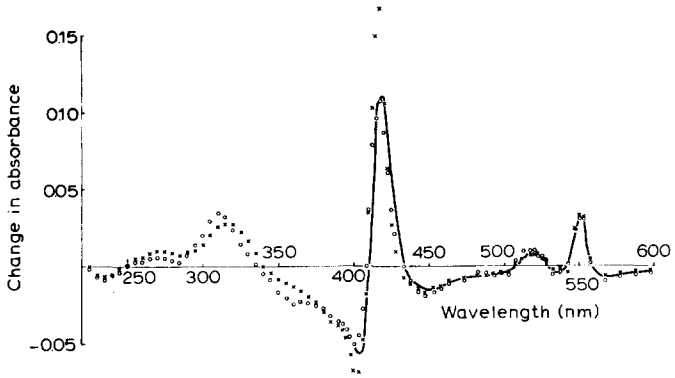

Fig. 1. Changes in absorption in the range 225–600 nm produced by delivering a pulse of radiation to a solution containing $8.4 \cdot 10^{-5}$ M ferricytochrome *c* and 10^{-1} M sodium formate (buffered to pH 9.2 with 10^{-2} M borate). ×, immmediately after e_{aq}^- and CO_2^- have reacted (≈ 1 ms); ○, after completion of the subsequent slow reaction (0.5 s); —, absorption changes produced on reduction by dithionite of an amount of ferricytochrome *c* equivalent to that reduced by the pulse ($1.75 \cdot 10^{-5}$ M cytochrome *c*).

theit reactions with cytochrome *c* and with each other it was estimated that 90 % of the e_{aq}^- and CO_2^- reacted with cytochrome *c*. It was found by giving repeated pulses that in these solutions hydrated electrons react at equal rates with oxidized and reduced cytochrome *c*. If it is assumed that CO_2^- also reacts equally with oxidized and reduced cytochrome *c*, then if the combined yield of hydrated electrons and CO_2^- is $G = 6.5$ [9], where G is defined as the number of species produced per 100 eV of energy absorbed in the system, it follows that $G = 5.3$ of these species produce reduction of cytochrome *c*, so that $3.2 \cdot 10^3$ rad reduces $1.75 \cdot 10^{-5}$ M cytochrome *c* (i.e. about 20 % reduction). If CO_2^- does not react with reduced cytochrome *c*, then $3.2 \cdot 10^3$ rad reduces $1.85 \cdot 10^{-5}$ M cytochrome *c*. Absorbance differences produced by the reductions are shown in Fig. 1, together with the difference between the absorption spectrum of $1.75 \cdot 10^{-5}$ M oxidized and reduced cytochrome *c* obtained by addition of sodium dithionite to a solution like that used in the pulse radiolysis experiments. The presence of absorbing species resulting from the addition of the sodium dithionite itself restricted determinations in solutions containing dithionite to wavelengths above 400 nm. It can be seen that immediately after the hydrated electrons and CO_2^- have reacted the difference spectrum in the Soret region is different from what it is by 0.5 s. By 0.5 s the difference spectrum is indistinguishable from that produced by dithionite reduction. It can, therefore, be concluded that the ultimate spectroscopic changes produced by hydrated electrons and CO_2^- are the same as those produced by sodium dithionite to within about 10 %. In order to compare the effects produced by hydrated electrons and CO_2^-, the same solutions were on the same day on the one hand deaerated by bubbling with argon and on the other hand saturated with N_2O so as to convert hydrated electrons into CO_2^- through the reactions:

$$e^-_{aq} + N_2O \xrightarrow{+H^+} N_2 + OH \qquad (1)$$

$$OH + HCOO^- \longrightarrow H_2O + CO_2^-. \qquad (2)$$

Absorbances were then measured at a number of wavelengths both immediately after the hydrated electrons and/or CO_2^- had reacted and at 0.5 s, with as little disturbance to the apparatus as possible. Results normalized to the same dose are shown in Table I from which it can be seen that both the wavelengths where the maximum absorptions take place (417.5 and 552.5 nm) and the change in absorbance at the maxima are the same for the two solutions, showing that hydrated electrons and CO_2^- produce indistinguishable effects. From the errors in the measurements, these effects must, therefore, be the same to well within 10 %.

TABLE I

Change in absorbance at various wavelengths produced by delivery of a pulse (approx $.3 \cdot 10^3$ rad) to a solution containing $9.3 \cdot 10^{-5}$ M cytochrome *c* and 0.1 M sodium formate at pH 9.2.

Wavelength (nm)	Absorbance ($\times 10^2$)			
	Argon saturated		N_2O saturated	
	Immediate	0.5 s	Immediate	0.5 s
410	2.12	2.52	1.85	2.19
412.5	7.47	6.00	7.89	5.94
415	13.6	8.86	14.2	9.36
417.5	15.6	10.3	15.9	10.9
420	12.0	9.24	12.6	9.10
422.5	7.73	6.91	6.85	6.40
547.5	1.94	1.77	2.00	1.79
550	3.09	2.90	2.94	2.92
552.5	3.46	3.29	3.48	3.33
555	2.50	2.24	2.28	2.02

Ferricytochrome *c* possesses a weak absorption band around 695 nm which is believed to be caused by a charge transfer interaction between the sulphur atom of Methionine 80 and the ferric iron atom. This absorption is lost when the iron atom is reduced to the ferrous state. Determination has now been made of the change in absorbance at 695 nm produced by delivering single pulses of fast electrons (approx. $3 \cdot 10^3$ rad) to deaerated solutions containing $2 \cdot 10^{-4}$ M cytochrome *c* and 0.1 M sodium formate buffered to pH 9.2 with 10^{-2} M borate buffer. Absorbances at 695 nm were found to increase after the pulse owing to the formation of hydrated electrons ($\varepsilon_{695\,nm} = 1.8 \cdot 10^4\ M^{-1} \cdot cm^{-1}$) and then to decrease within about 1 μs in a first order manner with a half-life corresponding to the rate of reaction of hydrated electrons with cytochrome *c* ($1.8 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ in this solution [1]). The absorbances of the solutions after disappearance of the hydrated electrons were consistent with a concurrent decrease in the absorption at 695 nm of the cytochrome *c* molecule itself. Following these changes, slower first-order decreases in absorbance were found to take place over several microseconds, the half-life being equal to that for reaction of CO_2^- with cytochrome *c*, showing that this reduction too was accompanied by a decrease in cytochrome *c* absorption at 695 nm. Still further first-order decreases then took place over some hundreds of milliseconds, following which the absorbance showed little change over seconds. In separate experiments it was found that the absorbance at 695 nm increased again over periods of minutes.

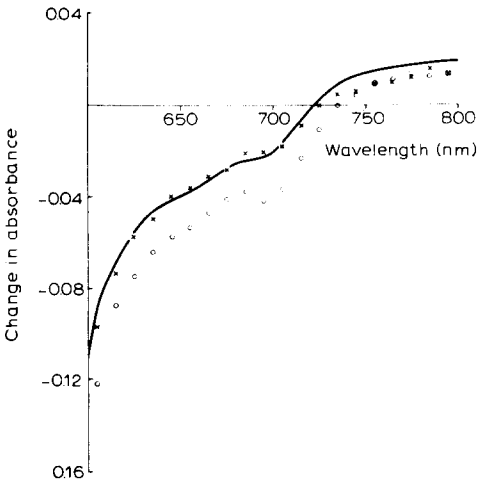

Fig. 2. Changes in absorption in the range 600–800 nm produced by delivering a pulse of radiation to a solution containing $8.4 \cdot 10^{-5}$ M ferricytochrome *c* and 10^{-1} M sodium formate (buffered to pH 9.2 with 10^{-2} M borate). ×, immediately after e^-_{aq} and CO_2^- have reacted (≈ 1 ms); ○, after completion of the subsequent slow reaction (0.5 s); —, absorption changes produced on reduction by sodium dithionite of an amount of cytochrome *c* equivalent to that reduced by the pulse ($1.9 \cdot 10^{-5}$ M cytochrome *c*).

Measurements have been made in the wavelength range 600–800 nm of the difference between the absorbance of cytochrome *c* solutions before the pulse and the absorbance (a) after hydrated electrons and CO_2^- have reacted but before the onset of the subsequent slow process and (b) after completion of the slow process (0.5 s). The solutions were like those used for Fig. 1. The dose delivered in each pulse was $3.5 \cdot 10^3$ rad which on assumptions like those above corresponds to the reduction of $1.9 \cdot 10^{-5}$ M cytochrome *c*. Absorbance differences produced by this reduction are shown in Fig. 2, together with the difference between the absorption spectrum of $1.9 \cdot 10^{-5}$ M oxidized and reduced cytochrome *c* obtained by addition of sodium dithionite. It can be seen that the absorbance differences after completion of the slow process differ from those obtained after reduction with dithionite. In order to test whether this might be due to errors in the spectrophotometric measurements, a solution containing $5 \cdot 10^{-4}$ M cytochrome *c* and 0.1 M sodium formate was reduced by sodium dithionite at pH 9.3, and the difference between the spectrum of the reduced solution and the original solution was measured using both the optical system of the pulse radiolysis apparatus and a laboratory spectrophotometer. The difference spectra in the range 600–800 nm were found to be identical, showing that in Fig. 2 the differences between reduction by radiation at 0.5 s and by dithionite must be real. It was found that saturation of solutions with N_2O produced no significant effect on the absorption changes in the range 600–800 nm produced by a pulse of radiation confirming still further that changes produced by CO_2^- are the same as those produced by hydrated electrons to within 10 %.

The rate constant for the slow first-order decrease in absorbance at 695 nm after reduction of cytochrome *c* (10^{-4} M) by CO_2^- at pH 9.2 was found to be $(11.5 \pm 2) s^{-1}$ at 18.4 °C. The activation energy was determined by the same method

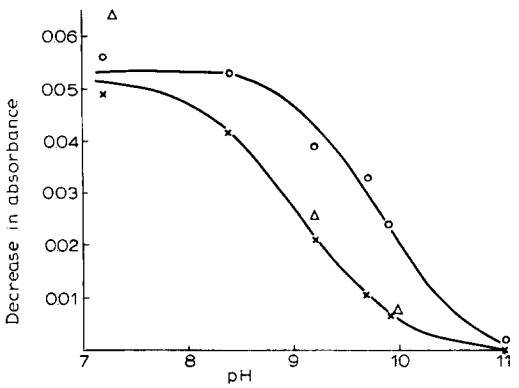

Fig. 3. Decrease in absorbance at 695 nm produced by delivering pulses of radiation to N_2O saturated solutions containing $8.4 \cdot 10^{-5}$ M ferricytochrome *c* and 10^{-1} M sodium formate at various pH. ×, immediately after CO_2^- has reacted (≈ 1 ms); ○, after completion of the subsequent slow reaction (0.5 s); △, change produced on reduction by dithionite of an amount of cytochrome *c* equivalent to that reduced by the pulse ($2.0 \cdot 10^{-5}$ M cytochrome *c*).

as previously employed for lower wavelengths [1], and found to be (80±12)kJ/mole.

The effect of pH on the magnitude of the absorbance changes at 695 nm was determined by giving $3.7 \cdot 10^3$ rad pulses to N_2O saturated $9.3 \cdot 10^{-5}$ M cytochrome *c* solutions containing 0.1 M sodium formate at various pH values. The results are shown in Fig. 3 which also shows absorbance changes produced in similar solutions on reduction by dithionite of an amount of cytochrome *c* equal to that reduced by the pulse. Using $2 \cdot 10^{-4}$ M cytochrome *c* solutions it was found at pH 9.9 that after decreases in absorbance like those in Fig. 3 the absorbance at 695 nm increased again over some minutes, and that the increase was more marked than at pH about 9.2. The effect of pH on the change in absorbance at 417.5 nm, the wavelength showing the biggest difference in absorbance between oxidized and reduced cytochrome *c*, is shown in Fig. 4. In a separate series of experiments it was found that altering the pH in the range 10.9 to 12.6 produced little effect on the immediate change in absorbance at 417.5 nm.

It was not easy to determine the effect of pH on the rate of the slow first-order decrease in absorbance at 695 nm since little slow change occurred below pH about 8.5, and the amount of absorbance above pH about 10 was extremely small. Within this range however there seemed to be little effect of pH on the rate. A determination was made of the rate of the slow process at 417.5 nm. The solutions used were the same as those employed to obtain the results of Fig. 4, the cytochrome *c* concentration being 10^{-4} M. At each pH the slow change was found to follow good first-order kinetics except that above pH 10 there was evidence of a longer-lived component in addition to the main one. Results obtained at 19.5 °C are shown in Table II. For pH 9.2 the rate constant of $(10 \pm 1)s^{-1}$ is somewhat above the earlier value of 7.3 s^{-1} which is now superseded. It can be seen from Table II that there is little effect of pH on the rate below pH about 10, but that a distinct decrease in rate occurs in more alkaline solutions. In further experiments it was found that at a constant pH of 9.2 the rate constant for the slow change was independent of cytochrome *c* concentration in the range $2 \cdot 10^{-5}$–10^{-4} M.

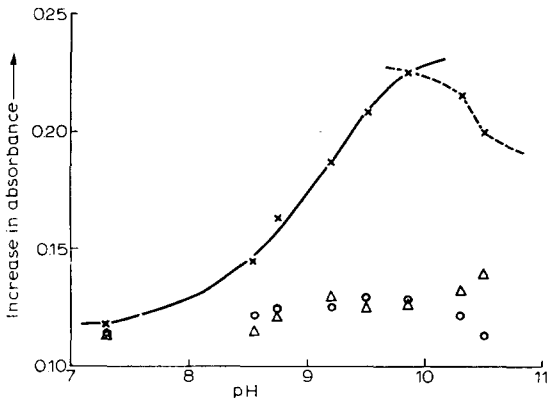

Fig. 4. Increase in absorbance at 417.5 nm produced by delivering pulses of radiation to solutions containing $9.0 \cdot 10^{-5}$ M ferricytochrome *c* and 10^{-1} M sodium formate at various pH. ×, immediately after e_{aq}^- and CO_2^- have reacted (≈ 1 ms); ○, after completion of the subsequent slow reaction (0.5 s); △, change produced on reduction by dithionite of an amount of cytochrome *c* equivalent to that reduced by the pulse ($1.9 \cdot 10^{-5}$ M).

TABLE II

RATE OF CHANGE IN ABSORBANCE AT 417.5 nm AFTER DELIVERY OF A PULSE TO SOLUTIONS CONTAINING CYTOCHROME *c* AT VARIOUS pH

pH	Rate constant (s^{-1})
8.75	14 ±5
9.2	10 ±1
9.5	10 ±1
9.85	8.6±0.9
10.3	4 ±1
10.5	5 ±1

DISCUSSION

The results in Fig. 1 and Table I show that in the range 225–600 nm the ultimate changes produced by hydrated electrons and CO_2^- are the same as each other and the same as the changes produced by sodium dithionite. This conclusion is in agreement with our previous one [1], and with independent experiments (Nilsson, K., personal communication), but differs from that reached by some other workers who have concluded that only 30–70 % [10], 50 % [7] or 70–80 % [8] of hydrated electrons produce changes in the haem moiety like those produced by dithionite, the rest presumably reacting at other parts of the molecule. Our conclusion is also at variance with the conclusion that certain organic free radicals produce ferrocytochrome *c* more effectively than hydrated electrons do [6]. The difference between these conclusions is attributed in part to differences in experimental conditions such as pH and ionic strength, and in part to the difficulty [8] of making reliable measurements at narrow bands in strongly absorbing solutions. There seems no reason to doubt that under the present conditions, and taking all experimental errors into account,

hydrated electrons, CO_2^- and dithionite all produce the same ultimate change in the range 225–600 nm and, therefore, all produce ferrocytochrome *c* in practically 100 % yield. Now at pH values close to 9 about half of the cytochrome *c* exists in the neutral form in which the sulphur atom of Methionine 80 is coordinated to the iron atom, possessing an absorption band at 695 nm, and half exists in the alkaline form in which the sulphur of methionine is displaced by another group, perhaps the ε amino group of Lysine 79, with no band at 695 nm [11]. Reduction by hydrated electrons and CO_2^- takes place at every pH at values which approach the diffusion-controlled rate [1], so that under normal experimental conditions the reduction is complete long before the two forms of cytochrome *c* would have time to interconvert [12–14]. It is therefore, evident that both hydrated electrons and CO_2^- (like dithionite [11]) will reduce both forms of cytochrome *c*.

The transient appearance of an abnormally absorbing form of ferrocytochrome *c* during reduction in alkaline but not neutral solutions [1, 11] has been explained [11] by representing the two forms of ferricytochrome *c* as Forms A and C, respectively (Fig. 5), normal ferrocytochrome *c* as Form F (independent of pH) and the unstable form of ferrocytochrome *c* as Form D. In very alkaline solutions reduction then proceeds by the rapid conversion of Form C to Form D followed by the slow process in which Form D eventually gives Form F. In neutral solution Form A gives Form F directly. At pH 9.2 both processes occur. If absorption at 695 nm is characteristic of a sulphur–ferric bond, the scheme also explains why at pH 9.2 the difference spectrum in the range 600–800 nm immediately after the hydrated electrons and/or CO_2^- have reacted is almost the same as after addition of sodium dithionite to the solution (Fig. 2).

This simple representation of the reduction in terms of Forms A, C, D and F does not however explain why in Fig. 2 the differences in spectrum at 0.5 s are about twice as great as immediately after reduction, since no further loss of 695 nm absorption should occur once Form A has given Form F. Now at 19 °C the slow loss of absorption at 695 nm proceeds at the same rate as the slow loss of absorption at

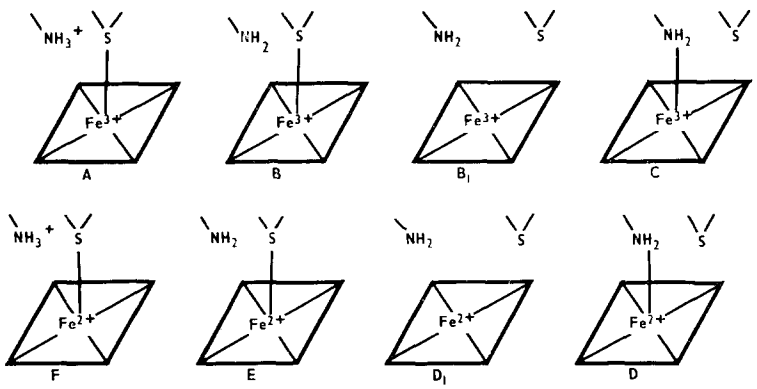

Fig. 5. Different forms of cytochrome *c*. Structures A and C are the forms of ferricytochrome *c* which are stable in neutral and mildly alkaline solutions, respectively. Structure F is the form of ferrocytochrome *c* which is stable in both neutral and mildly alkaline solutions. Structures B, B_1, E, D_1 and D are unstable forms of cytochrome *c* discussed in the text.

417.5 nm. Also the activation energy measured at 695 nm ((80 ± 12 kJ)/mole) is the same as at 417.5 nm (85 kJ/mole) [1]. This could not be because Form D disappears by reacting with Form A to give Form F (and Form C) because the rates of the slow processes are independent of cytochrome *c* concentration. It is, therefore, proposed that when Species D disappears it does not immediately give rise to Form F, but first yields another intermediate. The intermediate can then rapidly transfer an electron to unchanged neutral ferricytochrome *c* (Form A) so as to produce normal ferrocytochrome *c* (Form F). This transfer is consistent with the easy reducibility of the neutral form of ferricytochrome *c* as compared with the alkaline form [12]. The transfer would result in a loss of absorption in the 695 nm band which at pH 9.2 would be equal to that which had occurred in the direct reduction of Form A by hydrated electrons and/or CO_2^-. The electron transfer from the intermediate to Form A must proceed at a rate which is fast compared with the rate of conversion of Form D to the intermediate since the latter has to be rate determining. If the rate must be at least ten times the rate of conversion of Form D to the intermediate (which is 10 s^{-1} at 19 °C) then if the concentration of Form A at pH 9.2 is half the concentration of cytochrome *c* in the solution, i.e. is $5 \cdot 10^{-5}$ M, the rate constant for the electron transfer from the intermediate must be greater than or equal to $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. Rate constants of this order of magnitude have been recently observed for electron transfer from ferrocytochrome c_1 to ferricytochrome *c* and ferrocytochrome *c* to ferricytochrome c_1 [15]. If the intermediate is disappearing in our experiments by transferring an electron to Form A it follows that the rate of the unimolecular conversion of the intermediate to normal ferrocytochrome *c* (Form F) must be slow by comparison, and could, therefore, not have a rate constant of greater than 10 s^{-1}.

The slow increase in absorption at 695 nm during minutes after the pulse is explained by the re-formation of some Form A from other forms of ferricytochrome *c* following its depletion by reaction with hydrated electrons, CO_2^- and the intermediate. Although quantitative studies have not been made, the rate of this reaction is similar to the rates reported by others for the formation of the neutral form of cytochrome *c* from the alkaline form [12–14] and to the rate of the similar reaction which occurs when lyophilized cytochrome *c* is dissolved in water [16].

No evidence for a stage like the reaction of the intermediate with Form A has been seen in a previous study of the reduction of cytochrome *c* by sodium dithionite [11], and this must be because in that work all of the cytochrome *c* had been converted into the reduced form by the addition of dithionite so that no Form A would have remained in the solution for the intermediate to react with. Under such conditions the intermediate must convert directly into Form F.

The results of Fig. 3 are in accordance with our proposal of an intermediate formed in a slow process which rapidly transfers an electron to the neutral form of ferricytochrome *c*. So is our observation that at pH 9.9 the absorption at 695 nm increases markedly during a period of minutes after the pulse: this must be the re-formation of Form A from other forms of ferricytochrome *c* following removal of a substantial proportion of the A by hydrated electrons, CO_2^- and the intermediate. The similarity between the pH dependence of the initital changes at 417.5 nm (Fig. 4) and the initial changes at 695 nm (Fig. 3) suggests that both reflect the same coordination change in cytochrome *c* (p*K* about 9). Just as the kinetic behaviour of the 695 nm band altered above pH 10 (Table II), so does the trend in maximum absorption change

detected at 417.5 nm (Fig. 4): after increasing in the range pH 7 to 10, the 417 nm absorption begins to fall again above pH 10. This may reflect further changes in cytochrome *c* structure for which there is also ESR [17] and NMR [18] evidence.

In proposing a formula for the intermediate species, the first possibility to be considered is the previously proposed Structure E in which the sulphur atom of Methionine 80 is coordinated to the central ferrous ion, with the amino group unprotonated [11]. However if Structure E were to transfer an electron to Form A giving Form F it would be expected to be converted in the first instance into Form B which still possesses a sulphur–ferric bond, so that no loss of absorption at 695 nm would result. To account for the loss of 695 nm absorption during the electron transfer we, therefore, propose that the intermediate may have the Structure D_1 in which neither the amino group nor the methionine sulphur atom is coordinated to iron. Conversion of Structure D_1 into Form F (and the analogous conversion of Structure B_1 into Form A) may proceed via Structure E (or Structure B). It is emphasized, however, that the structures in Fig. 5 are merely hypothetical, and that other structures may also be consistent with our findings.

The rates of decay of both Structures D and D_1 are believed to be determined by concurrent conformation changes in the protein [1] rather than by simple changes in the inner coordination sphere of the ferrous atom, which would be expected to be much faster. The observed rates are within the range found previously for protein conformation changes in other systems [19, 20] ($0.1–10^4\ s^{-1}$). Similar slow conformation changes also probably occur in going from Form A to Form F at neutral pH, but these happen not to be reflected by absorption changes in the wavelength region studied. It has been pointed out [21] that the normal turnover time for cytochrome *c* in mitochondria is $500–1000\ s^{-1}$, much faster than the slow steps found here in aqueous solution. We believe this may be due to the conformational changes observed here not being fully achieved in the mitochondrion. If this is so it would follow that either in the oxidized form or the reduced form or both the structure of cytochrome *c* in mitochondria is different from what is it in aqueous solutions like those studied here.

ACKNOWLEDGEMENTS

We thank Drs C. Greenwood, K. Nilsson and M. T. Wilson for helpful discussions. We also thank Dr J. P. Keene and Mr B. W. Hodgson for their constant supervision of the accelerator and pulse radiolysis equipment.

This work was supported by grants from the Cancer Research Campaign and the Medical Research Council.

REFERENCES

1 Land, E. J. and Swallow, A. J. (1971) Arch. Biochem. Biophys. 145, 365–372
2 Pecht, I. and Faraggi, M. (1971) FEBS Lett. 13, 221–224
3 Wilting, J., Nauta, H. and Braams, R. (1971) FEBS Lett. 16, 147–151
4 Wilting, J., Braams, R., Nauta, H. and Van Buuren, K. J. H. (1972) Biochim. Biophys. Acta 283, 543–547
5 Lichtin, N. N., Ogdan, J. and Stein, G. (1971) Isr. J. Chem. 9, 579–582

6 Shafferman, A. and Stein, G. (1974) Science 183, 428–430
7 Lichtin, N. N., Shafferman, A. and Stein, G. (1973) Science 179, 680–682
8 Lichtin, N. N., Shafferman, A. and Stein, G. (1973) Biochim. Biophys. Acta 314, 117–135
9 Land, E. J. and Swallow, A. J. (1971) Biochim. Biophys. Acta 234, 34–42
10 Pecht, I. and Faraggi, M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 902–906
11 Lambeth, D. O., Campbell, K. L., Zand, R. and Palmer, G. (1973) J. Biol. Chem. 248, 8130–8136
12 Greenwood, C. and Palmer, G. (1965) J. Biol. Chem. 240, 3660–3663
13 Czerlinski, G. H. and Dar, K. (1971) Biochim. Biophys. Acta 234, 57–61
14 Wilson, M. T. and Greenwood, C. (1971) Eur. J. Biochem. 22, 11–18
15 Yu, C. A., Yu, L. and King, T. E. (1973) J. Biol. Chem. 248, 528–533
16 Aviram, I. and Schejter, A. (1972) Biopolymers 11, 2141–2145
17 Blumberg, W. E., Peisach, J., Hoffman, B., Stellwagen, E., Margoliash, E., Marchant, L., Tulloss, J. and Feinberg, B. (1973) Fed. Proc. 32, 469
18 Gupta, R. K. and Koenig, S. H. (1971) Biochem. Biophys. Res. Commun. 45, 1134–1143
19 Eigen, M. (1967) Fast Reactions and Primary Processes in Chemical Kinetics (Claesson, S., ed.), pp. 333–367, Interscience, New York
20 Hammes, G. (1968) Adv. Protein Chem. 23, 1–57
21 Takano, T., Kallai, O. B., Swanson, R. and Dickerson, R. E. (1973) J. Biol. Chem. 248, 5243–5255