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H/^{2}H ISOTOPE EFFECT IN REDOX REACTIONS OF CYTOCHROME *c*

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SUMMARY

The rate of reaction of ferro- and ferricytochrome *c* (C(II) and C(III)) with ferri- and ferrocyanide and of C(III) with O_2^- and CO_2^- was determined in H_2O and in ^2H_2O in the temperature range 5–35 °C. No isotope effect was evident in any of the reductions of C(III); the apparent energy of activation was identical in H_2O and ^2H_2O. An isotope effect with $k_{H_2O}/k_{^2H_2O} = 1.25$ to 1.85, depending on pH for instance was observed in the oxidation of C(II), in the slow phase of oxidation which involves conformational changes. An interpretation (supported by evidence from previous work) involving water molecules in the close vicinity of the reaction site on the protein is discussed.

INTRODUCTION

H/^{2}H solvent isotope effects on the rates $k_{H_2O}/k_{^2H_2O} = R$ of redox reactions have been critically discussed in Reynolds and Lumry's monograph [1], including the kinetics and suggested mechanisms in the case of iron ions and their complexes. When isotope effects are observed on changing the solvent from H_2O to ^2H_2O, they may serve in suggesting possible mechanistic pathways, involving at some stage changes in the position of solvent molecules or bonded protons. The absence of an isotope effect cannot however serve to exclude such involvement and indeed the magnitude of the effect and even whether R is greater, equal to or smaller than 1 may depend on the temperature for instance. There are detailed theoretical discussion [2, 3] of isotope effects on reaction rates in general, and further developments have recently been reviewed by Bell [4, 5].

With the reservations that are clear from the discussions in refs. 1–5 in mind, we report our results on $H_2O/^2H_2O$ isotope effects on redox reactions in the ferro-ferricytochrome-*c* (C(II)-C(III)) system. Such solvent isotope effects have been reported on components of the electron transfer chain in cellular systems [6]. In previous work, we studied the redox reactions of C(II)-C(III) with ferri-ferrocyanide ($Fe(CN)_6^{3-}$-$Fe(CN)_6^{4-}$) [7] and with O_2^-—O_2, as well as CO_2^- [8]. In these redox systems, our results were consistent with the assumption that water molecules

* Deceased.

in a defined reaction region on the protein play a role in determining the rates and mechanisms of the processes and participate in the structure of the transition state involving the enzyme and the substrate. Hence our interest in the possibility that an isotope effect, if found, may provide further assistance towards a mechanistic interpretation of such redox reactions of C(II)-C(III). In order to be able to make a strict comparison between results in H_2O and ^2H_2O we have carried out parallel experiments in both solvents under identical conditions. We used two main experimental techniques: (a) the method of fast generation of substrates in situ by means of pulse radiolysis [9] which we also used in refs. 7 and 8, and (b) temperature jump, which we used with $Fe(CN)_6^{4-}$-$Fe(CN)_6^{3-}$ as substrates [7]. In method (a), we mix reductant and oxidant in situ in times (< 1 μs) short compared to the chemical rates, and study processes far from equilibrium. In (b) we slightly disturb a system at equilibrium and study relaxation processes.

EXPERIMENTAL

Materials

C(III) (Sigma type III) was used without further purification. Its concentration was determined by measuring the absorption at 528 nm, using an extinction coefficient of $11.2 \cdot 10^3$ M^{-1} cm^{-1} [10].

We used Na_2HPO_4 and $K_3Fe(CN)_6$ (B.D.H.), $NaH_2PO_4 \cdot H_2O$ and $K_4Fe(CN)_6 \cdot H_2O$ (Mallinckrodt), $NaClO_4 \cdot H_2O$ (Fluka), *tert*-butanol (Merck, contained no impurities detectable by ultraviolet spectroscopy) and ethanol (Fluka). All these were of analytical grade and were used as supplied, as was O_2 (Israel Oxygen Center). N_2O and Ar (Matheson) were freed of oxygen by bubbling through a solution containing vanadous ions prepared by in situ reduction of Fluka purum grade $NaVO_3$ with zinc amalgam prepared from B.D.H. A.R. zinc and Frutarom analytical grade mercury.

The ^2H_2O used was Merck's Uvasol, 99.75 % ^2H_2O. The ordinary water used was triply distilled.

Apparatus

The Varian linear accelerator of the Hebrew University, the cell and the optical and electronic systems were described elsewhere [11]. The pulse produces the primary species: e^-_{aq}, H atoms and OH radicals; these react further as described below. Pulse lengths of approx. 1 μs were used.

Spectra of solutions were taken on a Cary 14 spectrophotometer, pH measurements on a digital pHM52 of Radiometer.

The temperature jump apparatus was constructed by Messanlagenstudiengesellschaft, m.b.h., Göttingen.

Procedures

Solutions were deaerated by sweeping with argon or saturated with N_2O or O_2, in large glass irradiation syringes equipped with capillary joint tapers for automatic renewal of solutions. Irradiations were carried out immediately after deaeration. Only one pulse was delivered to a given aliquot of solution.

Absorbed dose per pulse was determined routinely using the spectrum of the

hydrated electron, by pulsing $1 \cdot 10^{-2}$ M aqueous ethanol at pH $\cong$ 9.5, taking $\varepsilon_{578\,nm} = 1.06 \cdot 10^4\ M^{-1}\ cm^{-1}$ and $G(e_{aq}^-) = 2.75$ molecules/100 e.V. [12]. Doses used ranged from 350 to 1000 rad per pulse (equivalent to 10^{-6} M–$3 \cdot 10^{-6}$ M of e_{aq}^-). To ensure pseudo-first order conditions, the concentration of C(II) or O_2^- produced by the pulse were approx. 10 times lower than the concentrations of the initially present $Fe(CN)_6^{3-}$ or C(III). The kinetics of the C(III) $\rightleftarrows$ C(II) change was followed at 550 nm.

The temperature of the solution was usually 20$\pm$1 °C. When studying temperature effect, the temperature was kept within $\pm$0.5 °C of the specified value by using a thermostated cell.

The pH was adjusted to 7.2–7.4, by adding 2 mM of phosphate buffer, or to 9.2–9.4, by adding Na_2HPO_4. Oscilloscope traces were analyzed by transferring the data to punched 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. Cyber digital computer.

When two stages of a process could be separated (at basic pH) [7] the slow stage was analyzed first. The optical density of this stage was extrapolated by the computer program to zero time. The extrapolated value was taken as the optical density of the end of the fast stage.

The solutions for the temperature-jump measurements were not deaerated. The jump was from 21.6 to 25 °C. The relaxation times were derived from the change of the light transmitted by the solution at 550 nm, recorded on a Tektronix 549 storage oscilloscope, and photographed by means of a Polaroid camera.

RESULTS

(*a*) *Reduction of C(III) by* O_2^-

O_2^- reduces C(III) with a rate constant of $0.5 \cdot 10^6$–$1 \cdot 10^6\ M^{-1}\ s^{-1}$ (at neutral pH) or $1.1 \cdot 10^5$–$2.5 \cdot 10^5\ M^{-1}s^{-1}$ (at pH $\cong$ 8.5) [8, 13–16]:

$$O_2^- + C(III) \rightarrow C(II) + O_2 \qquad (1)$$

O_2^- was produced in oxygen saturated solutions containing 0.1 M NaHCOO (at neutral pH), or 0.067 M NaHCOO and 0.029 M Na_2HPO_4 (at basic pH), by the following reactions:

$$e_{aq}^- + O_2 \rightarrow O_2^- \qquad (2)$$

$$H + O_2 \rightarrow HO_2 \qquad (3)$$

$$HO_2 \rightleftharpoons H^+ + O_2^- \qquad (4)$$

$$OH + HCOO^- \rightarrow H_2O + CO_2^- \qquad (5)$$

$$H + HCOO^- \rightarrow H_2 + CO_2^- \qquad (6)$$

$$CO_2^- + O_2 \rightarrow CO_2 + O_2^- \qquad (7)$$

Reactions 2–7 are fast enough to be finished a few microseconds after the pulse, and thus the only radical which reacts with C(III) is O_2^- (the pK of reaction 4 being 4.9 [17]).

TABLE I

RATIO R IN THE REDUCTION OF C(III) AND THE OXIDATION OF C(II)

The ratio $R = k_{H_2O}/k_{2H_2O}$ in the reduction of C(III) by O_2^-, CO_2^- and $Fe(CN)_6^{4-}$, and in the oxidation of C(II) by $Fe(CN)_6^{3-}$ in the temperature range of 5–35 °C.

Oxidant/reductant	pH	Ionic strength μ (salt present)	R
O_2^-	7.4	0.1 (HCOONa)	1.0 ±0.1
O_2^-	8.5	0.1 (HCOONa)	1.0 ±0.1
CO_2^-	7.4	0.1 (HCOONa)	1.0 ±0.1
$Fe(CN)_6^{4-}$*	7.3	0.1 (phosphate)	1.0 ±0.1
$Fe(CN)_6^{3-}$**	9.3	0.1 (phosphate)	1.0 ±0.1
$Fe(CN)_6^{3-}$**	7.4	0.2 ($NaClO_4$)	1.0 ±0.1
$Fe(CN)_6^{3-}$†	9.3	0.1 (phosphate)	1.25±0.1††
$Fe(CN)_6^{3-}$†	7.4	0.2 ($NaClO_4$)	1.7 ±0.2††
$Fe(CN)_6^{3-}$†	7.3	0.007 (phosphate)	1.5 ±0.2††
$Fe(CN)_6^{3-}$†	7.3	0.1 (phosphate)	1.5 ±0.2††
$Fe(CN)_6^{3-}$*	7.3	0.1 (phosphate + $K_4Fe(CN_6)$	1.85±0.2††

* Measured by temperature jump.
** Fast stage of oxidation.
† Slow stage of oxidation.
†† The differences between the rate constants, k_{H_2O} and k_{2H_2O}, are significant on a 95 % confidence level or better.

Two solutions were prepared for each concentration of C(III) ($1.5 \cdot 10^{-5}$–$2.5 \cdot 10^{-5}$ M), one in H_2O, and one in ^2H_2O. In both solvents the kinetics were pseudo-first order, the rate being proportional to C(III) concentration. In both solvents, the second-order rate constants were: $(5 \pm 0.3) \cdot 10^5$ $M^{-1}s^{-1}$, at neutral pH, and: $(2.0 \pm 0.2) \cdot 10^5$ $M^{-1}s^{-1}$, at pH = 8.5 (Table I). Changing the temperature at pH = 9.2–9.4 in the range 5–35 °C did not bring about an isotope effect.

(*b*) *Reduction of C(III) by* CO_2^-

CO_2^- is known to reduce C(III) faster than O_2^- [8, 14]:

$$CO_2^- + C(III) \rightarrow C(II) + CO_2 \tag{8}$$

CO_2^- was produced in N_2O saturated solutions containing the same constituents as the solution prepared for studying the reduction by O_2^-. N_2O scavenges the hydrated electrons and transforms them to OH radicals:

$$N_2O + e_{aq}^- + H_2O \rightarrow N_2 + OH^- + OH \tag{9}$$

The OH radicals and H atoms are transformed to CO_2^- anion radicals according to reactions 5 and 6. Reaction 8 was pseudo-first order, the rate being proportional to C(III) concentrations ($1.5 \cdot 10^{-5}$–$2.5 \cdot 10^{-5}$ M). No significant difference was found between the results in H_2O and ^2H_2O (Table I). The observed second-order rate constant was $(7 \pm 0.5) \cdot 10^8$ $M^{-1}s^{-1}$ at neutral pH, and $(5.0 \pm 0.6) \cdot 10^8$ $M^{-1}s^{-1}$ at basic pH.

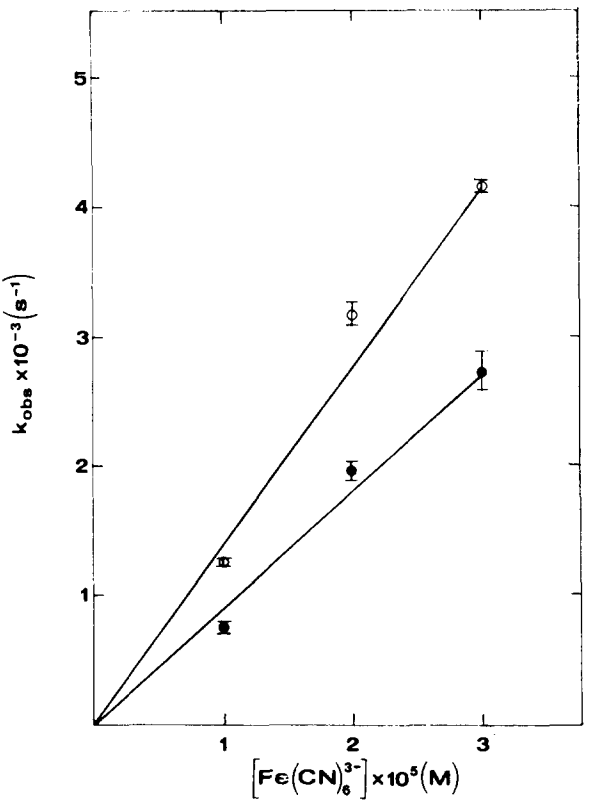

Fig. 1. Pulse radiolysis. Observed rate constant for the oxidation of C(II) by $Fe(CN)_6^{3-}$ as a function of $Fe(CN)_6^{3-}$ concentration. pH, 7.3; μ, 0.007 (phosphate buffer). [C(III)], $1.5 \cdot 10^{-5}$–$2.5 \cdot 10^{-5}$ M. ○–○, in H_2O; ●–●, in ^2H_2O.

(c) Oxidation of C(II) by $Fe(CN)_6^{3-}$ studied by pulse radiolysis

When C(II) is produced in situ by the reduction of C(III) by hydrated electrons and H atoms [11, 18] in the presence of $Fe(CN)_6^{3-}$, it is possible to follow its re-oxidation by $Fe(CN)_6^{3-}$ [7]. Solutions contained $1 \cdot 10^{-5}$–$4 \cdot 10^{-5}$ M C(III), 0.1–0.4 M *tert*-butanol and $K_3Fe(CN)_6$ in the concentrations indicated in Fig. 1. At pH 7.0–7.3 the solutions contained 2 mM (ionic strength $\cong$ 0.007), or 40 mM ($\mu \cong 0.1$) phosphate buffer. At basic pH the solutions contained 0.033 M Na_2HPO_4.

The in situ production of C(II) was completed within 2 μs after the pulse. The OH radicals generated by the pulse were scavenged by the *tert*-butanol present. The only reaction observed thereafter was reaction 10:

$$C(II) + Fe(CN)_6^{3-} \rightarrow C(III) + Fe(CN)_6^{4-} \qquad (10)$$

The kinetics were pseudo-first order, the rate being proportional to the concentration of $Fe(CN)_6^{3-}$. The results at neutral pH are shown in Fig. 1. The second order rate constants derived from the slopes are: $(1.4 \pm 0.1) \cdot 10^8 M^{-1}s^{-1}$ in H_2O and $(9.2 \pm 1.0) \cdot 10^7$ $M^{-1}s^{-1}$ in ^2H_2O. The ratio of these two values is 1.5 ± 0.2 (Table I).

The value of the rate constant in H_2O is in good agreement with the formerly

observed value at a similar ionic strength ($\mu \cong 0.007$) [7]. A value of $R = 1.5 \pm 0.2$ was also observed in an ionic strength of approx. 0.1 at the same pH (Table I).

C(III) has a pK_a of 9.3 [19, 20]. Therefore, it exists at pH 9.2–9.4 in two different conformations in about equal concentrations. As was already observed [7, 21], when C(III) is reduced at this pH by e_{aq}^-, two populations of C(II) are produced: one having the native conformation of C(II) at this pH (which is far from the pK_a of C(II)-12 [20, 21]) the second having a conformation similar to that of the basic conformation of C(III). Both kinds of C(II) are oxidized by $Fe(CN)_6^{3-}$, but with different rates. The kinetics of the oxidation are therefore biphasic at basic pH.

The rate of the slower stage of oxidation is the same as of the oxidation at neutral pH in the same ionic strength, and therefore this stage is the oxidation of the native conformation of C(II).

The rate of the faster stage is greater by about two orders of magnitude and was attributed to the oxidation of non-relaxed C(II) [7].

The rate constant of the slower stage at pH 9.2–9.4 is different in H_2O and ^2H_2O, and is: $(6.9 \pm 0.5) \cdot 10^6$ $M^{-1}s^{-1}$ in H_2O and $(5.5 \pm 0.5) \cdot 10^6$ $M^{-1}s^{-1}$ in ^2H_2O. The result in H_2O is in good agreement with the result at the same ionic strength (0.1) of ref. 7. The ratio of the rate constants $k_{H_2O}/k_{^2H_2O}$ is lower than at neutral pH: 1.25 ± 0.1. On the contrary, the rate constant of the faster stage (the oxidation of non-relaxed C(II)) is the same in H_2O and in ^2H_2O and is: $(2.6 \pm 0.2) \cdot 10^8$ $M^{-1}s^{-1}$ (Table I). In the presence of 0.2 M $NaClO_4$, at pH $\cong$ 7.3, the situation is similar to that at basic pH: the oxidation proceeds in two stages [7]. Again, the faster stage does not show an isotopic effect on its kinetics, whereas the slower stage does show an isotopic effect, namely: $k_{H_2O}/k_{^2H_2O} = 1.7 \pm 0.2$. Temperature change in the range 5–35 °C had no effect on k_{ox}, both in H_2O and in ^2H_2O. Temperature, therefore, has no effect on R. The Arrhenius activation energy for the oxidation of C(II) by $Fe(CN)_6^{3-}$ in H_2O is known to be zero (ref. 7, and references therein). We observed a similar result for this reaction in ^2H_2O.

(d) Oxidation of C(II) by $Fe(CN)_6^{3-}$ and reduction of C(III) by $Fe(CN)_6^{4-}$, as studied by temperature jump

In order to study the isotope effect on the reduction of C(III) by $Fe(CN)_6^{4-}$, as well as on the oxidation of C(II) by $Fe(CN)_6^{3-}$, we performed some experiments on a temperature jump apparatus. The solutions contained a total concentration of cytochrome *c* in the range $1.5 \cdot 10^{-5}$–$7.5 \cdot 10^{-5}$ M, 8 mM $K_4Fe(CN)_6$ and 30 mM phosphate buffer pH $\cong$ 7.2 ($\mu \approx 0.1$). In these solutions, reaction 10 reaches an equilibrium state, where the following expression prevails [22]:

$$\tau^{-1} = 2\,k_{ox}[\overline{C(II)}] + k_{red}[Fe(CN)_6^{4-}]_0$$

k_{ox} is the oxidation rate constant, k_{red} is the reduction rate constant, $[Fe(CN)_6^{4-}]_0$ is the initial concentration of the ferrocyanide anion, τ is the observed relaxation time, and $[\overline{C(II)}]$ is the equilibrium concentration of C(II). $[\overline{C(II)}]$ was calculated from the absorption spectrum of the solutions, using the peaks of the reduced protein at 550 and 520 nm, and extinction coefficient of ref. 10 for the oxidized and reduced molecules. The sum [C(II)]+[C(III)] derived by this procedure was compared with the total concentration calculated using the absorptions at the isosbestic points at 526.5 and 541.75 nm [10].

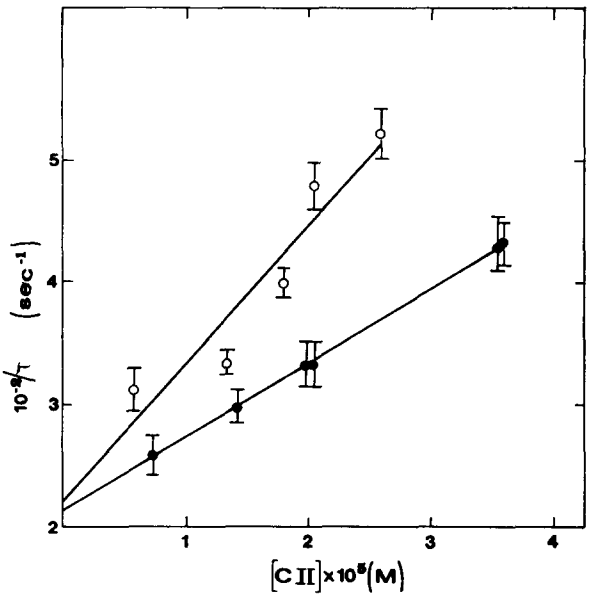

Fig. 2. Temperature jump. Inverse value of relaxation time, τ^{-1}, as a function of C(II) concentration. pH, 7.3, (30 mM phosphate buffer). $[K_4Fe(CN)_6]$, 8 mM; μ, 0.1. Total cytochrome concentration: $1.5 \cdot 10^{-5}$–$7.5 \cdot 10^{-5}$ M. ○–○, in H_2O; ●–●, in ^2H_2O.

The results are shown in Fig. 2 from which it is seen that k_{ox} derived from the slopes of the straight lines obtained, is different in H_2O as compared to ^2H_2O. On the other hand, k_{red} which is calculated from the intercept of these lines is the same in H_2O and in ^2H_2O.

The values of k_{ox} are: $(5.7\pm1) \cdot 10^6$ $M^{-1}s^{-1}$ in H_2O, and $(3.1\pm0.1) \cdot 10^6$ $M^{-1}s^{-1}$ in ^2H_2O. The value of k_{red} is: $(2.6\pm0.1) \cdot 10^4$ $M^{-1}s^{-1}$. The ratio $k_{ox}^{H_2O}/k_{ox}^{^2H_2O}$ is 1.85 ± 0.2 (Table I).

DISCUSSION

Kihara and McCray [6] found a water solvent $H/^2H$ isotope effect on the linked redox reactions of C-type cytochromes in photosynthetic whole bacteria, and in mammalian mitochondria. They emphasize that they find R close to $\sqrt{2} = 1.4$ in all cases independent of temperature. They report R values of 1.25–1.56. In the following, we shall see that the exact factor $\sqrt{2}$ has probably little real significance. They also showed the specific necessity of water being present. The reactions are greatly slowed down by exchanging water for e.g. ethylene glycol.

Melander [2, p. 126] discusses how, depending on the details of the mechanism, R may take different values bigger or smaller than 1, when the solvent isotope effect is connected with e.g. protonation in the intermediate or transition state [3–6]. Reynolds and Lumry [1] show that in the case of redox reactions intermediate protonation, or the making or breaking of O-H bonds in the rate-determining step need not occur and isotope effects may arise from bridge water molecules and solvent rearrangement in the reaction path. R may also change with temperature.

In our case the isotope effect was observed only in the oxidation of ferrocytochrome *c*, and not in the reduction of ferricytochrome. Moreover, only the "slow" reaction stage in which the fully relaxed C(II) molecule participates shows the effect.

To discuss the details of the process, we recall the following. As Theorell and Akesson showed C(III) has a pK of approx. 9.3 [19, 20]. Below this pH, C(III) in its "neutral" form has a more closed structure at the heme crevice, than in its "alkaline" form. Also the sixth ligand of iron changes from Met-80 in the neutral to lysine (probably 79) in the alkaline form. By contrast, C(II) has no pK in this region, up to pH $\cong$ 12 and has an even more tightly closed structure than neutral C(III). In our detailed investigation of the redox reactions of C(III)/C(II) with $Fe(CN)_6^{4-}$/$Fe(CN)_6^{3-}$ we confirmed in kinetics [7] the previous findings from equilibrium studies [23] that the relatively small reagents, $Fe(CN)_6^{3-,4-}$, bind specifically to identical reaction site(s) on C(II) and C(III). Related kinetic observations were also made by Cusanovich et al. [24, 25]. From studies of ionic effects on rate [7] we have concluded, as also in the case of reduction by O_2^- and CO_2^- [8] that the kinetically effective binding site is probably a group of lysines, (not including 79 [7, 26], close to but not extending into the heme crevice.

We could observe kinetic phenomena connected with structural and configurational change (involving also water structure in the close neighbourhood of the protein) by two approaches [7, 8]: (1) at pH < 9.3, we rapidly reduced C(III), by a pulse of e_{aq}^-. At such pH, C(III) is present as a mixture of two populations, the neutral and alkaline form. Before relaxation to its fully closed C(II) conformation, a non-relaxed form of C(II) is present, which still has the open configuration of alkaline C(III). This non-totally relaxed C(II) is reoxidized to C(III) by $Fe(CN)_6^{3-}$ with a fast rate, $k \cong 3 \cdot 10^8$ $M^{-1}s^{-1}$, while fully relaxed C(II) is reoxidized slower, $k \cong 7 \cdot 10^6$ $M^{-1}s^{-1}$ ($\mu = 0.1$).

Is the greater velocity in the case of non-relaxed cytochrome connected with the sixth ligand? We attempted an answer to this by examining: (2) the effect of added perchlorate ion on the reaction rate with $Fe(CN)_6^{3-}$. The change in the sixth ligand causes spectroscopic changes at $\cong$ 695 nm in C(III). Perchlorate does not produce this effect but, nevertheless, produces the fast stage of non-relaxed C(II) reoxidation and does so with an apparent p$K \cong$ 7.4 effective kinetically but not spectroscopically. Butler et al. [15] found such kinetic effect with p$K \cong$ 7.4 in the reduction of C(III) by O_2^-, as also found by us [7]*. We concluded [7, 8] that the effect is connected with water structure and protonation in the reaction region close to the cytochrome protein, caused by the effect of perchlorate ion on water structure.

Land and Swallow observed directly the relaxation of non-relaxed C(III) produced in situ at pH $\cong$ 9 [21], and also at pH $\cong$ 7 in the presence of high concentrations of alcohols [27]. The alcohol effect, again, might be connected with its influence on the structure of water and of the protein.

The rate of oxidation of C(II) is thus closely connected with structural changes from the closed configuration of C(II) to the more open configurations of C(III) in neutral and even more in alkaline solution. It appears also to be connected with the participation of water molecules in the structure involved in the reaction region.

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* The p$K \cong$ 7.4 in the case of C (III) reduction by O_2^- was recently the subject of some doubts [16].

The evidence now obtained from the solvent isotope effect is consistent with the assumption that participation of water molecules in reaching the transition state for electron transfer from C(II) does introduce a kinetic factor which affects the rate [1].

The kinetic isotope effect that we observe appears only in the oxidation of relaxed C(II). It appears neither in the oxidation of non-relaxed C(II), nor in the reduction of C(III) by O_2^-, CO_2^- and $Fe(CN)_6^{4-}$.

This indicates that the formation of the transition state for the oxidation of the relatively closed molecule of relaxed C(II) involves some rearrangement of water molecules, and/or bonds of H(^{2}H) atoms, which affects the overall rate. In contrast, such a rearrangement does not affect the overall rate of the reactions of the relatively open molecules of non-relaxed C(II) and of C(III).

As Reynolds and Lumry [1] pointed out, in the transition state for electron equivalent transfer the formation of the activated complex requires (ligand and) solvent reorganization so that the electronic energy of the reactants activated complex equals that of the products activated complex. On going from the reduced form such solvent reorganization becomes part of the rate determining process. Since k_{ox} does not depend on temperature, neither in H_2O nor in ^2H_2O, an entropic rather than an enthalpic factor causes the slower rate of reaction in ^2H_2O.

Using transition state theory, we calculated the entropy of activation ($\Delta S^{\neq}$), both in H_2O and in ^2H_2O. $\Delta S^{\neq}$ is more negative in ^2H_2O by 0.5–0.8 e.u. (depending on R), than it is in H_2O. Such an entropic factor is consistent with a difference in solvent reorganization in the two solvents.

To clarify some points arising from the temperature jump experiments, we note: the rate determining step must be the same forward and backward. If the forward reaction has an isotope effect, and the backward does not, the equilibrium constant has the same isotope effect as the forward rate constant.

The ratio relaxed C(II)/nonrelaxed C(II) in the pulse radiolysis experiments depends on the pH. The non-relaxed form is oxidized faster than it can relax, as shown in ref. 7.

In the temperature jump, the non-relaxed form does not appear. C(III) resembles relaxed C(II) more closely than it resembles non-relaxed C(II). There is no real equilibrium between the two forms, because only the relaxed is stable.

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