EFFECT OF PHYSICAL FACTORS ON REACTIONS OF HORSE-RADISH PEROXIDASE COMPLEXES WITH REDUCED CYTOCHROME *C*

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ABSTRACT This investigation concerns the effect of certain physical factors—viscosity, dielectric constant, ionic strength, and temperature of the medium—on the reaction of hydrogen peroxide and ferrocytochrome *c* in the presence of the enzyme horse-radish peroxidase. From study of the effects of viscosity and dielectric constant, it was concluded that the reaction between the secondary complex of hydrogen peroxide and enzyme on the one hand and ferrocytochrome *c* on the other is controlled by diffusion in media of high viscosity and by electrostatic effects at low viscosities. With respect to ionic strength, the data at pH 4.7 indicated a dipole-dipole interreaction. The temperature dependence of the over-all reaction had a Q_{10} of 1.25.

INTRODUCTION

Studies of the influence of various physical factors on the reactions of certain enzyme and heme-protein systems have been carried out in the past (1–7). These investigations have provided information concerning diffusion control, the size of the active sites, and the energies and entropies of activation. This paper presents the results of an investigation of the influence of certain physical factors on the reaction of hydrogen peroxide and ferrocytochrome *c* in the presence of the enzyme horse-radish peroxidase (HRP). The physical factors specifically studied are the viscosity, dielectric constant, ionic strength, and temperature of the medium in which the reactions occur.

Beetlestone (8) recently reported on the oxidation of cytochrome *c* by cytochrome *c* peroxidase. The dependence of the reaction rate on the ionic strength at pH 7.0 suggested that the reaction could occur at oppositely charged sites on the two proteins; each site would have an active area of approximately 5 A^2. The present investigation indicated that the reaction between the HRP-H_2O_2 secondary

compound and cytochrome *c* occurred between two oriented dipoles whose active areas are approximately the size of the iron atom in the heme group at pH 4.7.

THEORY

Mechanism of HRP Action. HRP catalyzes reactions between hydrogen peroxide and a suitable electron donor. Two electrons are transferred during the over-all reaction. In the presence of one-electron donors, a considerable amount of experimental data indicates that the reaction may occur as follows (9, 10):

$$\begin{matrix} e - p_1 - p_2 & x & p_1 \\ E & + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S_{\mathrm{I}} \end{matrix} \tag{1}$$

$$\begin{matrix} p_1 & a & p_2 & a' \\ E \cdot S_{\mathrm{I}} + & AH & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H \cdot E \cdot S_{\mathrm{II}} + & A \end{matrix} \tag{2}$$

$$\begin{matrix} p_2 & a & & \\ H \cdot E \cdot S_{\mathrm{II}} + & AH & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + & A + \text{products} \end{matrix} \tag{3}$$

where the k's refer to the forward and reverse rate constants and the other lower-case symbols indicate instantaneous concentrations.

If a steady-state condition is assumed, if k_{-2} and k_{-3} are negligibly small, and if $x \gg a$ and $k_1 x > k_3 a$, the solution to the differential equation describing the reactions is

$$k' = \frac{1}{2te} \ln \frac{a_0}{a} \tag{4}$$

where

$$k' = \frac{1}{1/k_2 + 1/k_3} \tag{5}$$

and a_0 is the initial donor concentration. For cytochrome *c*, k_1 is approximately equal to k_3 at low viscosities (11) but may differ considerably at high viscosities.

Two particular cases of equation (5) are of interest. If $k_2 \gg k_3$, it is seen from equation (4) that k' and k_3 are identical. Such an approximation is valid for the peroxidase reactions at 25°C in a medium at water viscosity, since Chance has shown that for many one-electron donors $k_2 \approx 70\, k_3$ (12).

At higher viscosities, the inequality between k_2 and k_3 may not hold. In fact, it is quite reasonable to assume that $k_2 \approx k_3$ in extremely viscous media in which the two reactions are controlled by diffusion. The reason for this is as follows. Both reactions involve a complex reacting with ferrocytochrome *c*. The two complexes are approximately the same size; therefore, the diffusion rates and, hence, the

encounter rates should be equal providing both reactions are controlled by diffusion. Under these conditions, $k' = \frac{1}{2}k_3$. At intermediate values of viscosity, k' should be a function of both k_2 and k_3.

Electrostatic Interactions. Amis (13), as well as Glasstone and associates (14), have derived theoretical expressions for specific rate constants as a function of the dielectric constant and ionic strength of the medium for various electrostatic interactions. They have theoretically investigated and experimentally verified their expressions for ion-ion, ion-neutral molecule, ion-dipole, and dipole-dipole reactions. In all these instances, log k varies linearly with the reciprocal of the dielectric constant (D). The slope of straight line obtained when log k is plotted against $1/D$ may be either negative or positive depending on the sign of the charge on the ion and the orientation of the dipole.

EXPERIMENTAL PROCEDURE

Chemicals. All experimental chemicals were obtained commercially. The HRP was obtained from the Sigma Chemical Company, St. Louis, as a powder. Solutions generally were prepared by dissolving approximately 30 to 40 mg of enzyme in 2 to 3 ml of 0.01 M phosphate buffer at pH 7.0. Stock solutions were kept in a refrigerated room at 0–3°C for 2 to 3 weeks without affecting the reaction rates. The concentration of each solution of HRP was determined spectrophotometrically by measuring the optical density of a dilute solution in a Beckman DU spectrophotometer at four wavelengths, namely, 403, 410, 420, and 430 millimicrons. The concentration was calculated by taking differences of the measured optical densities and dividing by the corresponding differences in millimolar extinction coefficients (9, 10).

Cytochrome *c* also was obtained from the Sigma Chemical Company. For the use of equation (4), the concentration is not needed, although an excess of hydrogen peroxide must be used. For this purpose, a rough estimate of the concentration of ferrocytochrome *c* was obtained by calculation from measurements of the optical density at 520, 535, and 550 millimicrons.

The cytochrome *c* was in the oxidized form and it was necessary to reduce it (15). Approximately 50 mg of cytochrome *c* was dissolved in 5 ml of 0.01 M phosphate buffer pH 7.0 containing a small amount of 5 per cent palladium asbestos (about 30 mg). The solution was gassed first for about 15 minutes with nitrogen, then for several hours with hydrogen, and finally for 15 minutes with nitrogen. The solution then was filtered twice with S and S sharkskin filter paper and stored in a stoppered container under a nitrogen atmosphere in a refrigerated room.

The hydrogen peroxide used was the certified reagent grade of the Fisher Scientific Company, Chicago, assayed at 3.53 per cent hydrogen peroxide by weight. Glycerol with a specific gravity of 1.249 at 25°C was obtained from Merck & Co., Inc., Rahway, New Jersey. Glycine (aminoacetic acid) was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Illinois, and was of cp grade.

Measurement of Viscosity and Dielectric Constants. The viscosity of the various solutions was measured by means of Cannon viscometers. The densities of the solutions were determined by first weighing an empty, clean, dry, 5 ml volumetric flask and then reweighing when it was filled to volume with the unknown solution.

Mixtures of glycerol and water were used to vary the viscosity of the solutions. After the viscosity of the glycerol solution was measured, the dielectric constant was obtained from standard tables of the dielectric constant *versus* temperature for various weight percentages of glycerol-water mixtures (16).

The dielectic constant of the medium was varied by using mixtures of potassium chloride and glycine. The data for dielectric constants in aqueous solutions of potassium chloride were obtained (17) from the equation

$$D_{KCl} = D_W - 10C \tag{6}$$

where D_W is the dielectric constant of water and C is the concentration of potassium chloride in moles per liter. The dielectric constant for glycine (18) was obtained from

$$D_{glycine} = D_W + 22.6C \tag{7}$$

where C is the concentration of glycine in moles per liter.

Procedure. The reactions were observed by measuring the changes in optical density at 550 millimicrons. Identical solutions were placed in both beams of light in a split-beam spectrophotometer. These solutions contained the enzyme and reduced cytochrome *c* but did not contain hydrogen peroxide. A small amount (0.02 ml) of a dilute solution of hydrogen peroxide was stirred into one of the cuvettes in about 1 second. The changes in optical density were recorded on a graphic level recorder. At the end of the reaction, the unreacted cytochrome *c* in the other cuvette was oxidized by adding substrate. The recorder trace returned to its original zero position. A reaction curve is shown in Fig. 1. The half-time for each reaction was determined from similar curves.

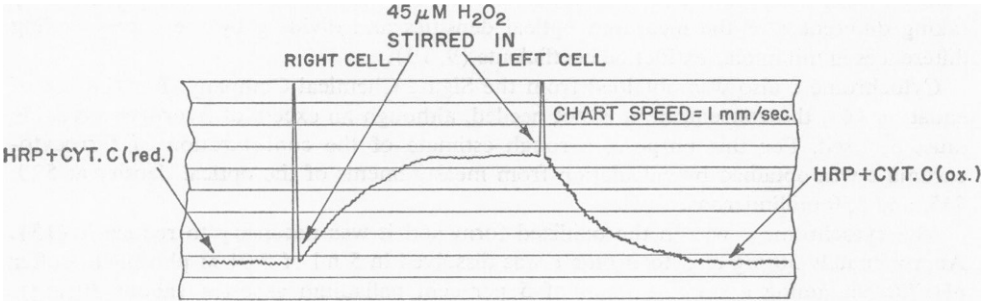

FIGURE 1 Typical reaction curve for cytochrome *c*. This measurement was made at 550 mμ. The vertical scale is about 0.006 OD per division. The distance between the peaks at which H_2O_2 is stirred in is 56 mm on the original chart.

These values then were averaged with other measurements obtained under similar conditions. Since the concentration of enzyme was known, k' was calculated directly from equation (4).

Auto-oxidation was a serious problem at low pH values. It was not practical to store reduced cytochrome *c* below pH 7. However, this auto-oxidation had a negligible effect on the experimental data reported, since the rate of oxidation catalyzed by peroxidase was so much greater than the auto-oxidation. Control experiments showed that corrections for auto-oxidation would have altered the computed value of the rate constants by

less than 5 per cent in the most unfavorable cases (very low pH). Inasmuch as other errors of measurement exceeded this, no such corrections were made.

RESULTS AND COMMENT

Effect of pH on k_3. The oxidation of cytochrome *c* by hydrogen peroxide in the presence of HRP is a reaction dependent on pH. The effect of pH on k_3 (at low viscosities, $k' \doteq k_3$) is shown in Fig. 2. Phosphate buffers (0.01 M) were used

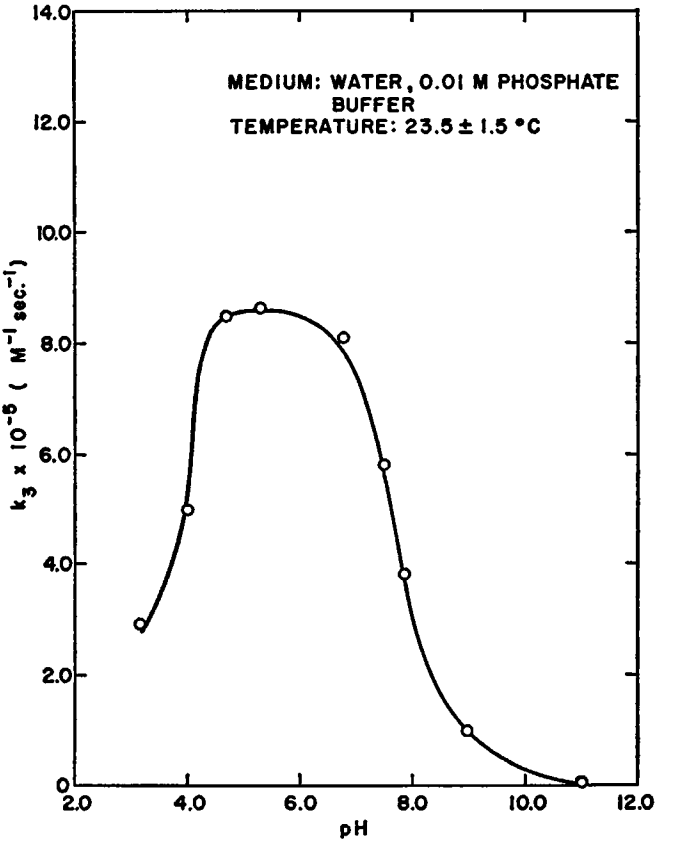

FIGURE 2 Effect of pH on k_3 for cytochrome *c*.

for the entire range of pH values.[1] The error in k_3 is approximately 10 per cent, so that the curve shown is an approximate one. The peak occurs in the pH region from 4.7–5.3. (It is a very broad peak. The entire region from pH 4.0–6.5 can

[1] These buffers are comparatively ineffective throughout a large part of this pH range; this did not seem important since no large pH changes occurred during the reactions. Furthermore, the measured values of k' were independent of the cytochrome *c* and peroxide concentrations throughout the entire pH range.

be regarded as a plateau in which the reaction rate changes by an amount comparable to the errors of measurement. A much greater precision of measurement would be necessary to resolve the details of this peak.) Subsequent measurements of the cytochrome *c* reaction were made in solutions buffered at approximately pH 4.7.

Beetlestone (8) measured the dependence on pH and ionic strength of the reaction rate of cytochrome *c* peroxidase. His published pH values covered the range 5.5–7.5, and as far as slope is concerned, his curve is similar to ours in this range.

Effect of Viscosity and Dielectric Constant on k'. The rate constant k' was determined for solutions containing varying amounts of glycerol up to approximately 90 per cent glycerol by weight. In order to facilitate mixing, the hydrogen peroxide was prepared at a viscosity identical with that of the solution being examined. The variations of k' with the fluidity are shown in Fig. 3. The dielectric constant, as well as the viscosity, is varying.

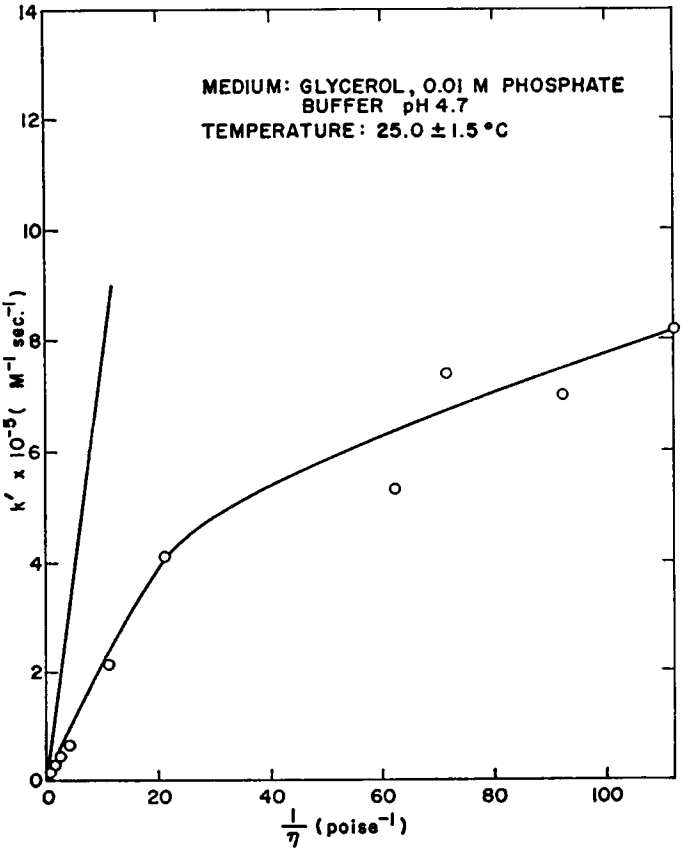

FIGURE 3 Effect of fluidity on k' for cytochrome *c*. The straight line passes through the origin and the first point. A diffusion-limited reaction should follow this line.

Two distinct regions in the graph are evident in Fig. 3. One extends over a range of fluidity from 110 (water) to 20 p^{-1} (50 per cent glycerol). The rate constant decreases only by a factor of 2 over this range. Hence, the reaction is certainly not entirely controlled by diffusion. From 20 to approximately 1 p^{-1}, the viscosity changes by a factor of approximately 20, while k' decreases also by a factor of 20. However, if it is assumed that $k_3 = 2k'$ at the highest viscosity measured, the rate constant k_3 would change only by a factor of 10. Therefore, even at viscosities corresponding to 90 per cent glycerol, the reaction characterized by k_3 may not be entirely controlled by diffusion. The changes observed in k_3 could be caused by electrostatic effects.

Effect of Dielectric Constant and Ionic Strength on k'. That an electrostatic effect is present is evident from the data presented in Fig. 4. The dielectric

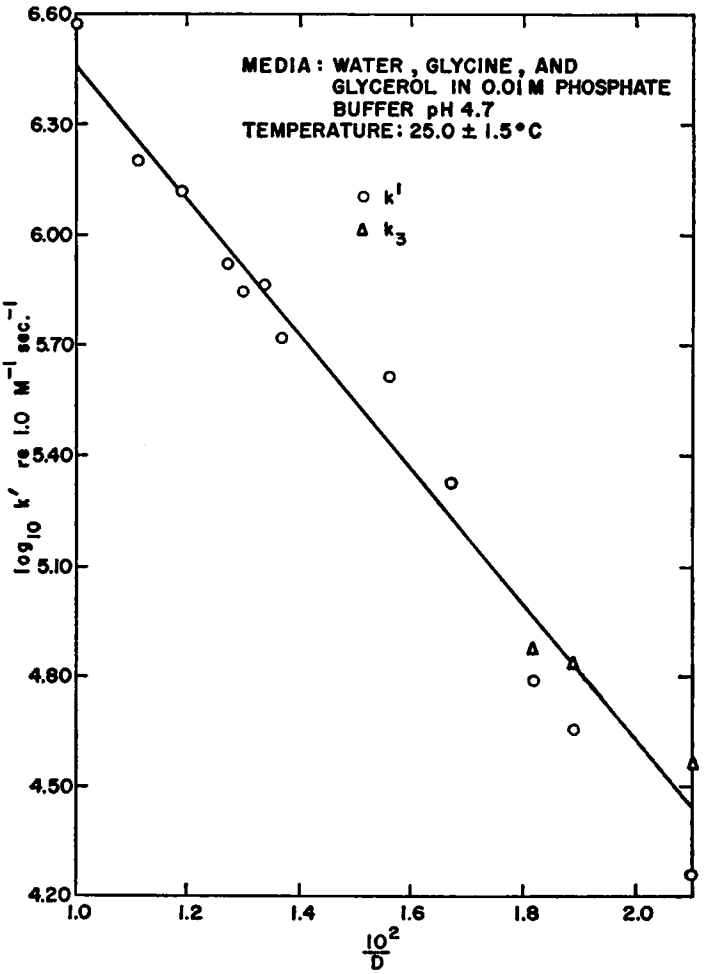

FIGURE 4 Effect of dielectric constant on k' for cytochrome *c*.

constant of the medium was varied by using different concentrations of glycerol to depress the dielectric constant from the value for water and by using glycine to raise it. During these measurements, the viscosity of the medium was varying also. The points corresponding to the three fastest reactions on the graph are for 1M, 0.5 M, and 0.25 M glycine. Over this range of concentrations of glycine, the viscosity varied from 1.22 cp for 1 M glycine to 1.13 cp for 0.25 M glycine at 18°C (19). At 18°C, the viscosity of water is 1.07 cp. Hence, it is seen that very little change occurred in the viscosity of the glycine solutions, and the changes in reaction rate may be ascribed to the effects of the dielectric constant.

Since the changes brought about by glycine can be attributed to a dielectric effect, a straight line was drawn through these points and the next few points, where the viscosity of the glycerol-buffer mixtures differed from that of water by less than a factor or two. If the line is extended, it can be noted that the points for the three most viscous reactions lie below the line. These three points represent k' in the three most viscous media. If one assumes that $k_3 = 2k'$ for the most viscous reaction, the three points representing k_3 will all be higher than those corresponding to k'. It can be seen that all the data on dielectric constants for k_3 fit the line extremely well. This result tends to discount a diffusion-controlled reaction for k_3 in media with viscosity less than 105 cp but assumes a diffusion-controlled reaction for k_2, even at 2 cp.

The line drawn through the points presented in Fig. 4 has a negative slope. From theoretical considerations, such a reaction could be one between two ions of like charge, between an ion and an oriented dipole, or between two dipoles. These can be distinguished by examination of the influence of the ionic strength.

According to the theory, the rate of reaction for two ions of the same sign should increase for increasing ionic strength when the dielectric constant remains unaltered. The ionic strength of the medium was raised by using different concentrations of potassium chloride. In order to keep the dielectric constant the same during the measurements, glycine was added to the solutions. It is possible to adjust the concentrations of potassium chloride and glycine so that the dielectric constant is equal to that of water. The viscosity of such solutions is not too different from that of water. Hence, any change in k' observed is due to the ionic strength of the solution. [Glycine, a zwitterion at pH 4.7, does not contribute to the ionic strength (20).]

Concentrations of potassium chloride and glycine were used that gave ionic strengths (μ) of 0.71, 0.36, and 0.19. Fig. 5*a* is a graph of log k_3 *versus* $(\mu)^{1/2}$. A point corresponding to the reaction rate in 0.01 M phosphate buffer at 25°C is also shown. Fig. 5*b* shows the variation of log k_3 at higher ionic strengths (2.0, 1.0, 0.5, 0.25) when only potassium chloride was used. The dielectric constant also varies, according to equation (6).

For low ionic strengths there is a slight tendency for k_3 to decrease, while at higher ionic strengths there is a slight increase in k_3 (Fig. 5*a*). The data will fit the straight line drawn within two standard errors. In any event, the data for ionic strength and

those for dielectric constants are conflicting for ions of like sign. Hence, it is likely that the reaction is one that does not require two ions. Likewise for an ion-dipole interaction, one would expect that an ionic-strength effect would be observed at low ionic strengths. Since a marked dependence on ionic strength has not been observed, it is concluded that the reaction is probably not one between an ion and a dipole. Similar independence of ionic strength and dependence on dielectric con-

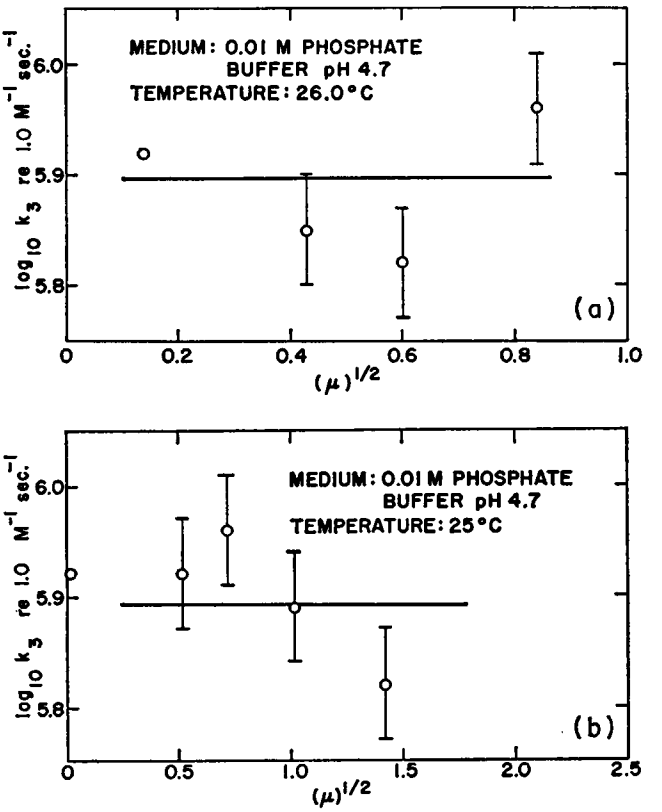

FIGURE 5 Effect of ionic strength on k_s for cytochrome *c*. (*a*) KCl + glycine. (*b*) KCl only.

stant have been reported for turnip peroxidase (21). Because of the more complex nature of the reactions they studied using two electron donors involving free radicals, the turnip peroxidase data are more difficult to interpret.

The final possibility for explaining the negative slope in Fig. 4 is a dipole-dipole reaction. In order to postulate such a reaction, it is necessary to be able to indicate the existence of dipoles on the enzyme (or rather complex II) and cytochrome *c* molecules. For HRP, there is a charge of plus one on the iron atom. At pH 4.7, the propionic acid group is ionized so that there is a negative charge because of

the carboxyl group (22). The possibility of other dipoles on both molecules also exists. For instance, the positive charge may reside on the iron atom while the negative charge may be located in the electron cloud surrounding the iron nucleus —a separation of approximately 1 A.

The iron in reduced cytochrome *c* is in the divalent state and has no net charge on the iron atom, but it is conceivable that instantaneously a charge is present on the iron atom. This would be a positive charge, since the nitrogen atoms adjacent to the iron atom in the heme group are more electronegative than is iron and hence tend to attract the electrons in the iron atom. The negative charges could be located as explained above for the peroxidase. In addition, it is possible that there are instantaneous dipoles induced by other charges or dipoles.

Beetlestone's data on effects in media of varying ionic strength all appear to have been obtained under conditions in which the bulk dielectric constant was also varying. These data can be interpreted as implying a dipole-dipole interaction just as our data can be. On the other hand, our data definitely conflict with the ion-ion interaction he postulates. The similarity of the pH dependence leads us to suspect that cytochrome *c* peroxidase might react with cytochrome *c* in a fashion analogous to that of horse-radish peroxidase, in spite of the large difference in the magnitude of the rates of reaction.

Further evidence for electrostatic and diffusion effects prevailing in the reaction of cytochrome *c* with HRP complex II is presented in Table I. A mixture of 72 per

TABLE I

VARIATIONS IN k' WITH DIELECTRIC CONSTANT AND VISCOSITY OF SUSPENDING MEDIUM

Medium	$k' \times 10^{-6}$ (M^{-1} sec.$^{-1}$)
Water	0.82
72 per cent glycerol + 1 M glycine	0.39
72 per cent glycerol	0.12

cent glycerol and 1 M glycine should have a dielectric constant of 78.5, the same as water. If only a dielectric constant effect were present, k' would be the same as in water. The measured value is intermediate between that found in water and that in 72 per cent glycerol without glycine. This observation is in accord with the previous assumption that k' depends on k_2 as well as k_3, and that k_2 cannot be neglected in the mixture of 72 per cent glycerol and 1 M glycine. Furthermore, the value of k_2 corresponding to this mixture of glycerol and glycine fits on the line joining the origin and the k_2 point at 1 p^{-1} in Fig. 3. This indicates that k_2 is controlled by diffusion at a viscosity corresponding to that of 72 per cent glycerol but that k_3 is not controlled by diffusion at this viscosity. In addition, one also may state that $k_2 \sim 3k'$ at a viscosity of 72 per cent glycerol.

Effect of Temperature on k'. The influence of temperature on k' for cytochrome *c* in three different media is shown in Fig. 6. The dielectric constant and the viscosity of the media are varying, as well as the temperature. The temperature was determined with a copper-constantan thermocouple placed in the cuvette.

From Fig. 6, the experimental energies of activation for the reactions in water, 50 per cent glycerol and 1 M glycine are 8, 8, and 10 kcal per mole, respectively.

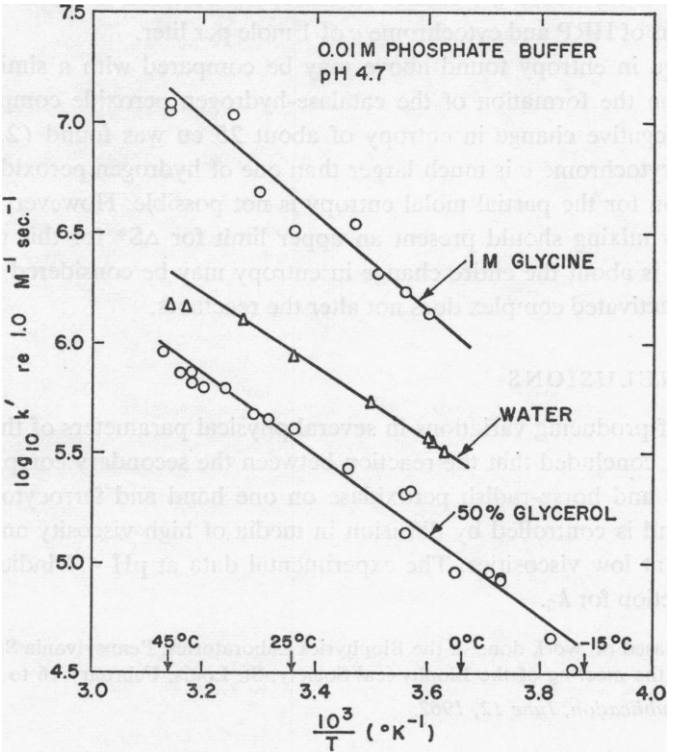

FIGURE 6 Effect of temperature on k' for cytochrome *c*.

Reactions with energies of activation of this magnitude could be controlled by diffusion; however, these numbers are typical of reactions which are independent of diffusion. (Data presented under effect of viscosity indicated that k' was independent of diffusion in water, 50 per cent glycerol, and 1 M glycine.)

The data in Fig. 6 indicate another effect controlling the reactions. Points for 50 per cent glycerol lie below those for both water and 1 M glycine. This may be attributed to the differences of the dielectric constants. The dielectric constant of 1 M glycine is 101.1, that of water is 78.5, and that of 50 per cent glycerol is 64.0. Thus, the different lines can be explained by dielectric effects.

A property of fundamental importance for reactions is the change in entropy that

accompanies the reaction. The reaction between the secondary peroxidase complex and cytochrome *c* has been shown to be independent of diffusion at all viscosities below those corresponding to 85 to 90 per cent glycerol. Therefore, the absolute-rate theory (14) may be applied to the reaction-rate constant k_3 for a calculation of the change in entropy during the reaction. From the temperature studies, the energy of activation is 8 kcal per mole in water. If k_3 equals 8×10^5 M^{-1} sec.$^{-1}$ at 25°C, ΔS^* equals -6 eu, where the change in entropy is referred to a standard state for concentrations of HRP and cytochrome *c* of 1 mole per liter.

The change in entropy found above may be compared with a similar measurement made on the formation of the catalase-hydrogen peroxide complex. For this reaction, a negative change in entropy of about 25 eu was found (2, 3). Since a molecule of cytochrome *c* is much larger than one of hydrogen peroxide, a theoretical calculation for the partial molal entropy is not possible. However, the loss of 7 eu caused by mixing should present an upper limit for ΔS^* for this reaction. The fact that this is about the entire change in entropy may be considered as an indication that the activated complex does not alter the reactants.

CONCLUSIONS

As a result of producing variations in several physical parameters of the suspending medium, it is concluded that the reaction between the secondary complex of hydrogen peroxide and horse-radish peroxidase on one hand and ferrocytochrome *c* on the other hand is controlled by diffusion in media of high viscosity and by electrostatic effects at low viscosities. The experimental data at pH 4.7 indicate a dipole-dipole interaction for k_3.

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