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Kinetic Study of the Oxidation of Ferrohemochrome by Molecular Oxygen*

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ABSTRACT: Oxidation of dipyridineferrohemochrome by molecular oxygen was studied in both aqueous and ethanol-benzene solutions containing an excess of pyridine. In the presence of a large excess of dissolved oxygen, oxidation in all the solutions studied follows first-order kinetics. In aqueous solutions, the rate data can be interpreted by a dual path mechanism. The main path involves formation of "oxyheme" followed by its redox decomposition. In ethanol-benzene solutions the rate decreases rapidly as the volume per cent of benzene is increased. The apparent activation energies are consistent with the proposed mechanism.

The hypothesis that hemoglobin and myoglobin owe their remarkable property of reversible oxygenation mainly to the hydrophobic environment of the embedded hemes in these molecules (Wang *et al.*, 1958; Wang, 1958) has recently found support in elegant X-ray work on these proteins (Perutz *et al.*, 1960; Kendrew *et al.*, 1960, 1961). In order to examine this problem further, it would be desirable to know explicitly the effect of the hydrophobic environment on the rate of oxidation of heme derivatives by molecular oxygen without possible complication by other structural factors of the protein. With this objective, the rate of oxidation of dipyridineferrohemochrome by oxygen has been studied in the present work in both aqueous solutions and various ethanol-benzene solutions containing an excess of pyridine. A possible mechanism is proposed to account for the kinetic data thus obtained.

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Experimental

Preparation of Aqueous Solutions. A 5×10^{-4} M hemin stock solution was prepared by first dissolving

32.6 mg of hemin (Eastman, recrystallized, 97+%) in 10 ml 1 M Tris solution at 70–80°. After cooling, the solution was diluted with 50 ml distilled water and enough 1 M HCl to adjust the *p*H to about 8.4, then was further diluted with water to a total volume of 100 ml.

The unstable dipyridineferrohemochrome solution was prepared by mixing the measured amounts of fresh hemin stock solution and pyridine, diluting the mixture with 0.1 M Tris buffer, and adjusting the final *p*H to the desired value with 1 M HCl, and then reducing the ferrihemochrome with hydrogen in the presence of platinum black. Hydrogen was chosen as the reducing agent because any excess of it can be removed easily after the reduction by evacuation or flushing with an inert gas. On the other hand, because of its tendency to attack the porphin ring of the pigment molecule, the reduction had to be controlled carefully. The following reduction procedure was found to be satisfactory.

Measured amounts of pyridineferrihemochrome solution and platinum black were put in a special glass container. The dissolved air was removed by passing through a sintered-glass gas-dispersion tube a stream of nitrogen which had been prewashed with alkaline pyrogallol solution. Then hydrogen was passed continually into the suspension through a hypodermic needle. The gas space in the container was flushed by nitrogen for about 5 minutes. In this way the excess of hydrogen was mostly swept out with nitrogen, and there still was enough hydrogen adsorbed on the platinum black to reduce the ferric complex. Up to this stage the solution retained its greenish-brown color characteristic of dipyridineferrihemochrome. But after standing 20 hours at room temperature the solution turned red and was ready for kinetic measurement. The spectra of the solution before and after reduction are shown in Figure 1. The spectrum of the reduced solution is characteristic of that of dipyridineferrohemochrome, as reported by Paul *et al.* (1953). Tris buffer, 0.1 M at the same *p*H as the ferrohemochrome solution and saturated with air at 1 atm, was used as the oxidizing solution.

Kinetic Measurements for Aqueous Solutions. The rates of oxidation of dipyridineferrohemochrome by dissolved oxygen in aqueous solution was followed by a "stopped-flow" apparatus constructed and described by Sturtevant and Spencer (1959).[1] A matched pair of 2-cc glass syringes, one containing the ferrohemochrome solution, the other air-saturated buffer, were connected by 1-mm capillary channels to a mixing chamber which fed the mixed solution to an observation chamber with quartz end plates 1.00 cm apart. The two syringes were operated by the same pushing bar to expel about 0.25 cc from each syringe each time, which is enough to flush out all the liquid remaining from the previous measurement. The flow rate

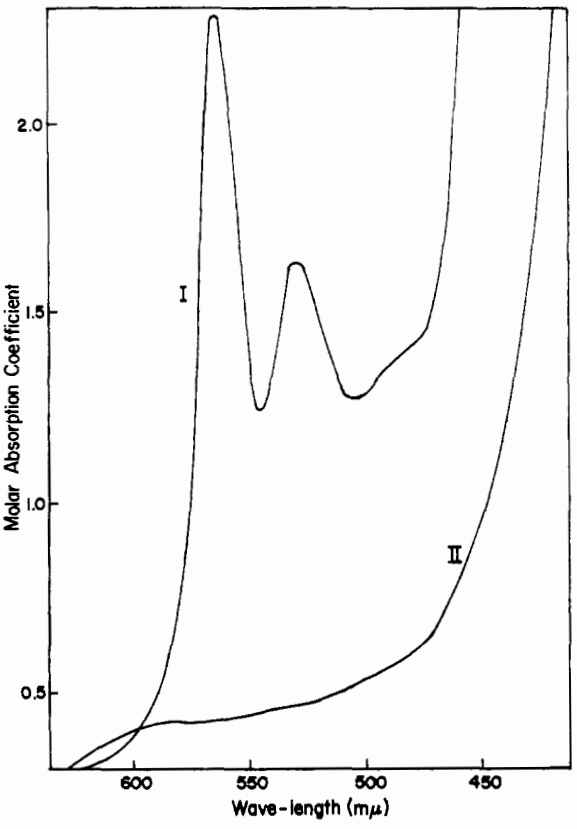

FIGURE 1: Absorption spectra of dipyridineferrohemochrome (curve I) and dipyridineferrihemochrome (curve II) in 0.1 M aqueous Tris buffer at *p*H 8.5. [Heme] = 5×10^{-5} M, [py] = 0.124 M.

of liquid in this apparatus is about 5 cc sec^{-1}, and average age of the mixed solution upon arrival in the observation chamber is 0.005–0.007 second.

The reaction was followed by measuring the optical density at 562 mμ with a Unicam SP 500 spectrophotometer which served as a monochromater and light source, a 1P28 photomultiplier tube, and a very stable power supply[2] and recording system. If the optical densities at time t and at the end of the oxidation reaction are denoted by D and D_∞, respectively, a plot of log $(D - D_\infty)$ versus t should give a straight line in case of first-order kinetics. The result of a typical measurement is illustrated in Figure 2.

Preparation of Nonaqueous Solutions. Dimethyl ester of hemin was prepared by the direct esterification of methanol and hemin in the presence of trifluoroacetic acid as catalyst. This was purified by repeated washing of its chloroform solution with cold 25% aqueous sodium carbonate solution and recrystallization from benzene. A stock solution was prepared by dissolving 8.5 mg of the dimethyl ester of hemin in a few drops of benzene, mixing with 10 ml of pyridine,

[1] The authors are indebted to Professor J. M. Sturtevant for kindly allowing them to use his apparatus and to Dr. C. Ho for his valuable advice on the calibration of the instrument.

[2] Model HV-3A, Technical Measurements Corp., New Haven, Conn.

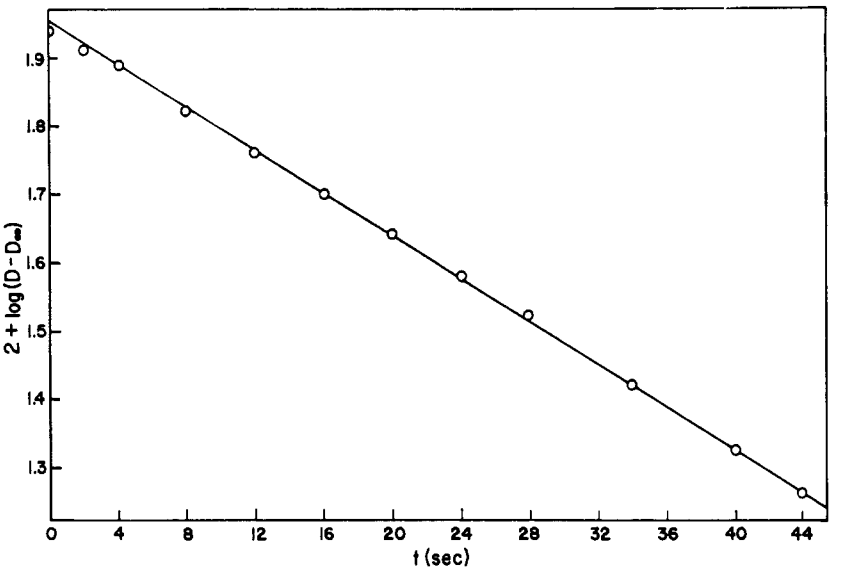

FIGURE 2: Oxidation of dipyridineferrohemochrome in 0.1 M aqueous Tris buffer at *p*H 8.0 and 25°. [Heme] = 1×10^{-4} M, [py] = 0.5 M.

and then diluting with benzene to a total volume of 25 ml. This solution was reduced by hydrogen in the presence of platinum black in the same way as described except that the nitrogen gas was further dried with concentrated sulfuric acid and solid KOH to remove water vapor.

The reduced stock solution was diluted with different proportions of benzene and ethanol just before kinetic measurements. The spectra of the dimethyl ester of dipyridineferro- and ferrihemochromes in ethanol are illustrated in Figure 3. Solutions of the dimethyl ester of heme in benzene containing 0.5 M pyridine were formed to obey Beer's law within ±0.5% in the concentration range from 0.16×10^{-4} to 0.42×10^{-4} M.

When pure benzene or pure ethanol was used as the solvent, the oxygen solution was prepared simply by passing oxygen gas through the solvent for about 1 hour. When mixtures of ethanol and benzene were used as solvents, the oxygen solutions were prepared by equilibration in a closed vessel to avoid possible change in solvent composition owing to fractionation. In either case the concentration of dissolved oxygen was determined spectrophotometrically by the iodine method, i.e., by oxidizing an equivalent amount of iodide to iodine in dilute H_2SO_4 in a closed vessel with manganous sulfate as catalyst and then determining the iodine concentraton by spectrophotometry (Shirley and Blinn, 1954).

Kinetic Measurements for Nonaqueous Solutions. Since the oxidation of the dimethyl ester of dipyridineferrohemochrome in ethanol-benzene mixtures is much slower than in aqueous solution, the rates were determined by carrying out the reactions in a thermostated, rubber-stoppered quartz cell and following the decrease in optical density at 555 mμ with a Cary Model 11 spectrophotometer. The quartz cell was encased in a thermostated metal block during the reaction. After the reaction, the dipyridineferrihemochrome solution showed the characteristic Soret absorption band of dipyridineferrihemochrome at 400 mμ in place of the 419-mμ band before oxidation. The α- β- and Soret bands of the ferrohemochrome can be regenerated by reduction with sodium dithionite solution. This shows that only the Fe(II) in the hemochrome was oxidized.

Measurement of the Dielectric Constant of Ethanol-Benzene Mixtures. The dielectric constant was measured with a capacitance bridge by the substitution method.[3] All the measurements were made at 200 kc and 25°.

Results and Discussion

Reactions in Aqueous Solution. Dipyridineferrohemochrome exists in monomeric form in its aqueous solutions containing an excess of pyridine,[4] and the solutions were found to obey Beer's law. The oxidation of dipyridineferrohemochrome by a large excess of molecular oxygen was found to follow first-order kinetics. This shows that the rate-determining step involves the oxidation of only one ferrohemochrome molecule. Since spectroscopic examination shows that only the Fe(II) of the ferrohemochrome was oxidized under the experimental conditions, one may conclude that the rate-determining step involves the transfer of only one electron. As an example, the straight line ob-

[3] The authors are indebted to Professor R. M. Fuoss for his permission to use the instrument and to Dr. J. Lind for his advice on these measurements. For details see Lind and Fuoss (1961).

[4] For the evidence in support of this statement, see the review by Phillips (1960).

tained by plotting log $(D - D_\infty)$ versus time in Figure 1 gives an apparent first-order rate constant of 3.9×10^{-2} sec^{-1} for 0.5 M aqueous pyridine solution at *p*H 8.0, 25°, and oxygen concentration of 2.66×10^{-4} M. The observed apparent first-order rate constant decreases as the pyridine concentration is increased. The measured values are summarized in Table I.

TABLE I: Summary of the Apparent First-Order Rate Constants (k) at Various Pyridine Concentrations.[a]

[Pyridine] (M)	1/[Pyridine] (M^{-1})	$k \times 10^2$ (sec^{-1})
0.0916	10.91	32.3 ± 1.6
0.124	8.1	22.1 ± 1.2
0.139	7.2	18.9 ± 2.7
0.186	5.4	17.1 ± 2.6
0.211	4.7	16.0 ± 0.55
0.248	4.0	13.6 ± 1.36
0.310	3.2	12.6 ± 0.29
0.372	2.7	8.2 ± 1.29
0.50	2.0	7.25 ± 0.92

[a] [Hemochrome] = 5×10^{-5} M, [Tris] = 0.1 M, *p*H = 8.5, $[O_2] = 2.66 \times 10^{-4}$ M.

The data in Table I can be interpreted by the assumption that the oxidation of dipyridineferrohemochrome by molecular oxygen takes place through the following dual-path mechanism. In the first path, an oxygen molecule replaces one of the pyridine molecules in dipyridineferrohemochrome to form an "oxyheme," which then undergoes decomposition to ferrihemochrome and O_2^- or HO_2. The subsequent reduction of O_2^- or HO_2 by three other dipyridineferrohemochrome molecules requires very small activation energy and hence is presumably not rate determining. In the second path, the electron transfer occurs during the collision of an oxygen molecule with the dipyridineferrohemochrome without prior substitution reaction. Denoting the reaction paths by

$$O_2 + \text{py-heme-py} \underset{}{\overset{K}{\leftrightarrows}} \text{py} + \text{py-heme-}O_2$$
$$\text{py-heme-}O_2 \xrightarrow{k_2'} \text{products} \qquad \text{py-heme-}O_2 \xrightarrow{k_1'} \text{products} \tag{1}$$

we have

$$K \equiv \frac{[\text{py}]x}{[O_2]\{[\text{heme}] - x\}} \approx \frac{[\text{py}]x}{[O_2][\text{heme}]}$$

where $[O_2]$ = molar concentration of dissolved oxygen, [heme] = molar concentration of ferrohemochrome, [py] = molar concentration of pyridine, and x = molar concentration of "oxyheme." The rate of oxidation is

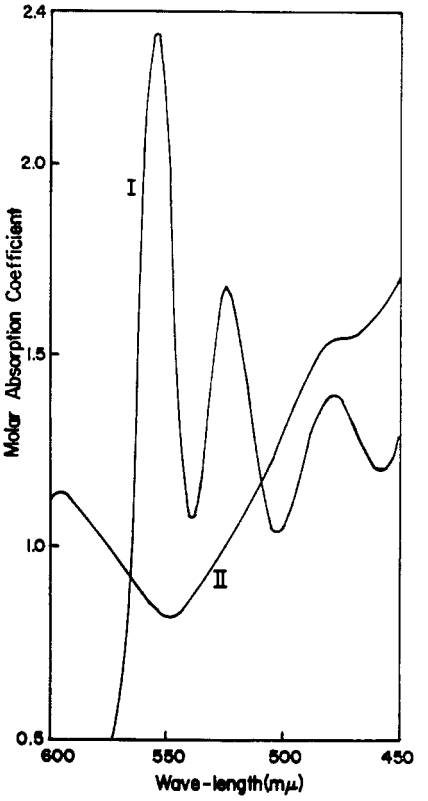

FIGURE 3: Absorption spectra of the dimethyl ester of dipyridineferrohemochrome (curve I) and of dipyridineferrihemochrome (curve II) in ethanol. [Heme diester] = 5×10^{-5} M, [py] = 0.5 M.

$$-\frac{d[\text{heme}]}{dt} = 4k[\text{heme}] = 4\{k_1'x + k_2'[\text{heme}][O_2]\}$$

or

$$-\frac{d \ln [\text{heme}]}{dt} = 4k = 4\left\{\frac{k_1'K}{[\text{py}]} + k_2'\right\}[O_2] \tag{2}$$

where k = the observed apparent first-order rate constant, k_1' = the apparent first-order rate constant for the first path, and k_2' = the apparent first-order rate constant for the second path. The factor 4 in equation (2) accounts for the fact that at the steady state the rate of the three successive oxidations following the rate-determining step have the same rate. The experimental values of k are plotted verses 1/[py] in Figure 4. The straight line in Figure 4 represents equation (2).

Figure 4 shows that the contribution of the second path to the observed reaction rate s very small at low pyridine concentrations. At very high pyridine concentrations, the dipyridineferrohemochrome may form loosely attached molecular adducts with additional pyridine molecules. Since these nonstoichiometric molecular addition compounds may have somewhat

TABLE II: Apparent First-order Rate Constant for the Oxidation of the Dimethyl Ester of Dipyridineferrohemochrome by Molecular Oxygen in Ethanol-Benzene Solutions.[a]

Solvent	D	$k \times 10^3$ (sec^{-1}) 9°	25°	35.5°	40°	50°	E_a (kcal/°C-mole)	$k_0 \times 10^{-5}$ (sec^{-1})
98.5% Ethanol–1.5% benzene	24.7	5.0	15.4		24	47	8.78	0.36
80% Ethanol–20% benzene	19.7	3.8	7.5		15	35	9.42	0.63
50% Ethanol–50% benzene	11.3	0.55	2.1		4.8	10	12.8	42
20% Ethanol–80% benzene	5.7		1.37	2.96	4.61	8.6	15.0	198

[a] [Pyridine] = 0.5 M, [O_2] = 4.3×10^{-3} M, $k \equiv k_0 \exp(E_a/RT)$.

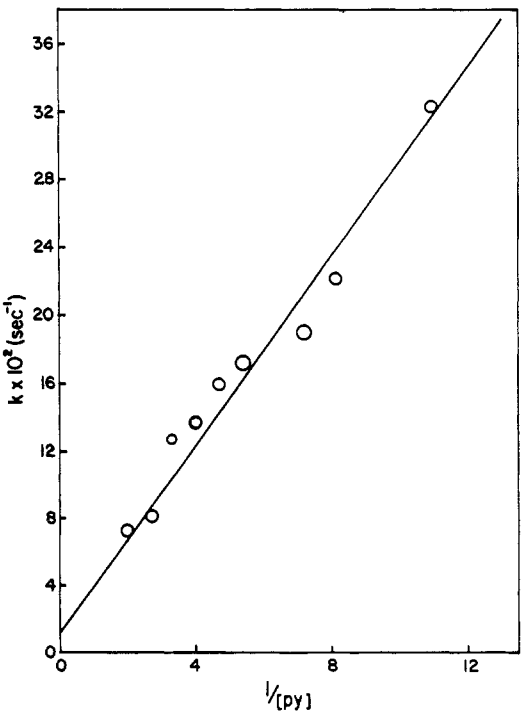

FIGURE 4: Apparent first-order rate constant (k) as a linear function of the reciprocal of pyridine concentration in 0.1 M aqueous Tris buffer at pH 8.5 and 25°. [Heme] = 5×10^{-5} M.

different kinetic behavior, quantitative studies becomes more complicated.

Effect of the Polarity of Solvent. Equal volumes of oxygen solution (saturated with oxygen at a partial pressure of 760 mm) and dipyridineferrohemochrome solution in ethanol-benzene mixtures were allowed to react. The measured apparent first-order rate constants are listed in Table II.

Assuming that in these dilute ethanol-benzene solutions the oxidation of dipyridineferrohemochrome takes place predominantly through the first path, i.e., formation of "oxyheme" followed by its decomposition to ferrihemochrome and O_2^- or HO_2, we would expect the corresponding rates to decrease as the volume per cent of benzene increases. This is certainly borne out by the data in Table II. It may be noted that even at sixteen times larger oxygen concentration the apparent first-order rate constant in 20% ethanol–80% benzene solution at 25° is more than 300 times smaller than that in 0.5 M aqueous pyridine solution. The rates in solutions using pure benzene as the solvent at 25° are too slow to be measured reliably by the present technique.

Measurements of the oxidation rate were also made at different oxygen concentrations. It was found that at [O_2] $\leq 1.3 \times 10^{-3}$ M the measured apparent first-order rate constant increases linearly with [O_2], but at higher oxygen concentrations the measured rate increases less rapidly. This gradual tapering off in rate at high oxygen concentrations may not be caused by saturation phenomenon, since spectroscopic examination of a series of these 0.5 M pyridine in ethanol-benzene solutions failed to establish the existence of any appreciable amount of the complex "oxyheme."

Temperature Dependence. The measured apparent first-order rate constants at four different temperatures for each of the four ethanol-benzene solutions studied are summarized in Table II. The apparent activation energies, E_a, were determined graphically in the usual way by plotting ln k versus $1/T$. The data show that the apparent activation energy in a less polar solvent is lower than that in a more polar solvent, whereas the change in apparent activation entropy is just the opposite. The physical meaning of the apparent activation energy, E_a, can be illustrated by the special case when the oxidation takes place mainly through the first path. Then equation (2) reduces to

$$k = \left\{\frac{k_1' K}{[\text{py}]}\right\} [O_2]$$

where [py] and [O_2] are constants for the whole series of measurements listed in Table II. Therefore

$$E_a \equiv -R \frac{d \ln k}{d\left(\frac{1}{T}\right)}$$

$$= -R \left\{ \frac{d \ln k_1'}{d\left(\frac{1}{T}\right)} + \frac{d \ln K}{d\left(\frac{1}{T}\right)} \right\} E_1' + \Delta H^\circ$$

where E_1' = activation energy for the redox decomposition of "oxyheme," and ΔH° = heat of formation of "oxyheme."

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Studies on the Mechanism of Biological Carbon Alkylation Reactions*

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ABSTRACT: Tuberculostearic acid (10-methylstearic acid) was isolated from *Mycobacterium smegmatis* grown in the presence of methionine-methyl-d_3. Mass spectrometry of the methyl ester of this fatty acid revealed the absence of any trideuterated species. A molecular peak corresponding to the dideuterated species, and less intense peaks corresponding to a monodeuterated species and a nonisotopic species, were observed.

An analogous experiment with ergosterol isolated from *Neurospora crassa* grown in the presence of methionine-methyl-d_3 gave essentially the same result. A molecular peak corresponding to the D_2 compound, but none corresponding to the D_3 compound, was found.

The classical studies of Keller *et al.* (1949) and duVigneaud *et al.* (1956) established that the methyl group of methionine could be transferred as a complete unit to various nitrogenous acceptors. By the use of methionine doubly labeled in the methyl group with both ^{14}C and deuterium, it was clearly established that deuterium atoms were not lost from the methyl group during transmethylation. Other investigations (Dewey *et al.*, 1954; Byerrum *et al.*, 1954; Sato *et al.*, 1957) have strengthened the idea that transfer of an intact methyl group is the normal course of biological transmethylation reactions.

In 1954, a new type of biological transalkylation[1] was postulated by Birch *et al.* (1954), and later demonstrated experimentally by Birch *et al.* (1957, 1958)—a reaction in which a methyl group of methionine is added to a presumably unsaturated carbon atom resulting in a methyl side chain. In more recent times several examples of this and slightly different carbon alkylation reactions have been discovered. These are summarized in Table I, with references to some of the known examples (see also, Mudd and Cantoni, 1964).

Since these carbon alkylation reactions are very

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[1] In this paper the terms "alkylation" and "transalkylation" refer to any process which results in a methylated or alkylated product, regardless of the over-all mechanism of reaction. It is intended to include classical transmethylation reactions as well as transmethylenations of the type studied here and of the cyclopropane fatty acid synthetase type.