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Pre-Steady-State Kinetics of Intermediate Formation in the Deuteroferriheme–Hydrogen Peroxide System†

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ABSTRACT: The pH dependence of formation of a peroxidatic intermediate from the reaction of deuteroferriheme with hydrogen peroxide has been determined for the region pH 8.7–10.1 from stopped-flow kinetic studies in which absorbancy changes are observed at heme monomer–dimer isosbestic points. Results are interpreted primarily in terms of the attainment of double "steady-state" concentrations of Michaelis–Menten complex I and peroxidatic intermediate I′. A linear correlation of observed first-order rate constants with α, the degree of dissociation of heme dimer, has been demonstrated and nonzero intercepts are obtained. Slopes and intercepts show a linear logarithmic dependence on pH which is interpreted in terms of HO_2^- participation both in the formation and subsequent (catalatic) decomposition of a peroxidatically active intermediate. General acid catalysis of intermediate formation is indicated from studies in phosphate, arsenate, and citrate buffer at pH 7.4–9.3. It is suggested that such catalysis may be responsible for anomalously high rates of H_2O_2 decomposition previously observed in phosphate buffer solution.

In a previous paper (Jones et al., 1974a), we reported on the kinetics of formation of the peroxidatic intermediate from the reaction of deuteroferriheme [chloro(dihydrogen 3,7,12,17-tetramethyl-2,18-porphinedipropionato(2$^-$))iron(III)] and hydrogen peroxide at pH 7.75 and 8.5 at 25 °C. The results obtained from stopped-flow spectrophotometric studies were interpreted in terms of the mechanistic model depicted in reaction 1,

$$M + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} I' \qquad (1)$$

wherein M denotes monomeric deuteroferriheme; I, the Michaelis–Menten complex; and I′, the peroxidatic intermediate analogous to compounds I of various oxidase enzymes, e.g., catalase (EC 1.11.1.6) reported by Chance et al. (1947, 1952). Values of kinetic parameters k_3 and $K_m(= (k_2 + k_3)/k_1)$ were calculated for reaction 1, employing data of Prudhoe (1971) for the pH and (heme) concentration dependence of the equilibrium concentrations of monomeric and dimeric heme at 25 °C.

Although this model modifies an earlier mechanism proposed by Portsmouth and Beal (1971) in recognizing mono-

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meric heme to be the predominantly active species in the reaction with H_2O_2, there are several questions which remain unanswered, one of which concerns the implied stoichiometric conversion of heme to a peroxidatic intermediate. Thus, if, under the conditions employed in the reaction with H_2O_2, the rate of deuteroheme monomer-dimer interconversion ($2M \rightleftharpoons D + H^+$) is rapid relative to the rate of change of solution absorbancy (Jones et al., 1974b), then, since α, the degree of dissociation of dimeric heme, increases with increasing stoichiometric concentration of heme, significant depletion of free heme via formation of a peroxidatic intermediate should result in a significant concurrent increase in the degree of dimer dissociation. For studies conducted in the presence of a large excess of hydrogen peroxide, this would be expected to produce an increase with time in the "apparent" pseudo-first-order rate constant manifest by curvature (increasing negative slope) in the time dependence of log $(A_t - A_\infty)$ (A denotes solution absorbancy). Contrary to such predictions, logarithmic plots remain linear over periods approaching two half-lives for the first-order decrease in optical density observed over various ranges of reactant concentration.

It should be noted also that reaction 1 does not recognize the (catalatic) role of intermediate in the decomposition of H_2O_2. This special case of peroxidatic activity may tentatively be depicted by expanding reaction 1 to show attack of substrate on I′, thereby producing, as depicted in reaction 2, a "stoichiometric mechanism" (Langford and Gray, 1965) formally identical to the "two-complex" mechanism of Kremer and Stein (1959) for the catalytic decomposition of H_2O_2 by ferric perchlorate. Reaction 2

$$M + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} I' \xrightarrow[+H_2O_2]{k_4} M + O_2 + H_2O \qquad (2)$$

resembles that proposed by Jones and Wynne-Jones (1962) and has the same form as the Kremer and Baer (1974) model for catalase action with the added feature that heme aggregation equilibria in solution are accommodated.[1] It modifies reaction 1 in that the catalatic reaction is not assumed to be negligible in the time required for attainment of a constant absorbance level in a given kinetic study. Further, in reaction 2, if the state corresponding to A_∞ (A at t_∞) for a particular study is interpreted as that of a double "steady state" in both I and I′, there are no implications of a near quantitative conversion of heme to I′. Indeed, under certain conditions this conversion may be far from stoichiometric, in which case significant deviations from linear first-order relations would not be expected at least over the range of one to two half-lives for the approach to A_∞. It seemed desirable, therefore, to examine the data for this system in terms of this modified model. With this view and a desire to expand the pH profile for the system generally, we were prompted to further explore the kinetics of this reaction under a wide range of experimental conditions.

Also of particular interest are kinetic effects peculiar to the nature and concentrations of selected buffer solutions. For example, an unexplained "specific effect" of phosphate buffer resulting in anomalously high rates of heme-catalyzed H_2O_2 decomposition has been previously reported (Jones et al., 1973a). In an attempt to elucidate such features, we have examined the influence of changes in concentrations of components of phosphate and other buffer systems on the rate of "intermediate" formation in the deuteroheme-H_2O_2 reaction.

[1] Terms employed in reaction 2 and in the consequent derivations depicted in equations 3–12 follow previous notations of Jones et al. (1974a,b) and are related formally to other notations used to represent mechanisms of hemoprotein and related enzyme-catalyzed reactions, e.g., catalase action. Thus, M which denotes monomeric heme corresponds to E (enzyme), H_2O_2 to S (substrate), I to ES or ES_I (enzyme–substrate complex), and I′ to ES_{II} or EO (catalase compound I or its analogue) (Chance et al., 1952; Kremer and Baer, 1974; Schonbaum and Chance, 1976).

Experimental Section

Deuteroferriheme was prepared by heating a ground mixture of 2 g of hemin (3× crystalline obtained from Nutritional Biochemicals Corp.) with 6 g of resorcinol in an oil bath at 150–160 °C for 45 min. The cooled crystals were washed with dry Et_2O (freshly distilled from $LiAlH_4$) until the washings were nearly colorless, and then dried in air. The product was then dissolved in 10 mL of $CHCl_3$ and, after removal of a small insoluble residue by filtration, crystallized by addition to a boiling solution containing 3 mL of saturated NaCl and 2 mL of concentrated HCl in 160 mL of glacial acetic acid. The crystals were washed with 50% aqueous acetic acid followed by distilled water, ethanol, and Et_2O. Recovered yield was 0.8 g. An alkaline pyridine hemochrome solution was prepared according to the method of Falk (1964). Strong absorption bands were found at 543 and 513 nm and a weaker peak was centered at 528 nm (lit. λ_{max}(A), 545 nm; λ_{max}(B) 515 nm; λ_{min} 530 nm; Falk, 1964). Optical density: A, 1.62 at 544 nm for a 6.6×10^{-5} M solution in a 1-cm cell; lit., A, 1.58 (assuming $\epsilon_M = 2.4 \times 10^4$; Falk, 1964).

Ultraviolet and visible spectra of deuteroferriheme solutions were determined at various concentrations in different buffered solutions using a Cary 15 recording spectrophotometer. Cuvettes of suitable path lengths were contained in cell holders through which was circulated water which was maintained at 25 ± 0.02 °C by a Sargent thermonitor water bath. Stopped-flow experiments were carried out as previously described (Jones et al., 1974a) with the following changes. A Durrum-Gibson D-110 stopped-flow spectrophotometer was employed in conjunction with a Tektronix oscilloscope equipped with a differential amplifier which gave an output proportional to the absorbancy of the solution in the spectrophotometer cuvette. Changes in absorbancy due to the formation of the peroxidatic intermediate from deuteroferriheme and hydrogen peroxide were examined at wavelengths corresponding to the isosbestic points for monomeric and dimeric deuteroferriheme at each respective pH.

Values of the observed first-order rate constant, k_{obsd}, were obtained from slopes of log $(A_t - A_\infty)$ vs. t for studies in which the peroxide–heme concentration ratios ranged from 10:1 to 200:1. At these relative concentrations, destructive oxidation of heme is negligible in the time of the absorbancy change from t_0 to t_∞ (Jones et al., 1973b). Over most of the range of peroxide concentrations employed, k_{obsd} is directly proportional to the initial peroxide concentration $[H_2O_2]_0$.

Carbonate, phosphate, arsenate, and citrate buffer components of reagent-grade quality were obtained from Mallinckrodt or Baker. Further extension of the study of kinetic effects of Brönsted acids was somewhat limited, first, through the requirement that the buffer be resistant to oxidation, which excluded the use of various amine salts and phenols and, secondly, through the limited solubility of heme at pH <7.2 which restricted the useful pK_a range for the acidic component of the buffer system and ruled out studies of effects of numerous carboxylic acids. All aqueous solutions were prepared using singly distilled deionized water.

Results and Discussion

The Heme Monomer–Dimer Isosbestic. Since attack of substrate hydrogen peroxide on monomeric heme is assumed to be slow relative to the rate of monomer–dimer intercon-

TABLE I: The pH Dependence of the Rate of Intermediate Formation at 25 °C (μ = 0.1).[a]

pH[b]	[T] × 10^5 (M)	α[c]	$k' \times 10^{-3}$ (M^{-1} s^{-1})[d]	$m \times 10^{-5}$ [e]	$b \times 10^{-3}$ [e]	log m	log b
10.08	0.50	0.0154	9.60	6.50	1.55	5.81	3.19
	1.0	0.0109	8.70				
	1.5	0.0089	7.20				
	2.5	0.0069	6.10				
9.70	0.50	0.0237	6.88	2.59	0.654	5.41	2.82
	1.0	0.0168	4.81				
	1.5	0.0137	4.23				
	2.5	0.0107	3.51				
9.05	0.125	0.0963	4.50	0.516	0.107	4.71	2.03
	0.25	0.0691	3.68				
	0.50	0.0494	2.64				
	1.0	0.0352	1.91				
	1.5	0.0288	1.61				
8.88	0.50	0.0597	2.56	0.405	0.137	4.61	2.14
	1.0	0.0426	1.85				
	1.5	0.0349	1.56				
8.69	0.25	0.1030	2.68	0.255	0.067	4.41	1.83
	0.50	0.0738	1.97				
	1.25	0.0473	1.26				

[a] λ 368.5 nm. [b] $[HCO_3^-] + [CO_3^{2-}] = 0.035$ M; NaCl added to give $\mu = 0.1$. [c] From Prudhoe (1971). [d] $k' = k_{obsd}/[H_2O_2]$. [e] From $k' = m\alpha + b$.

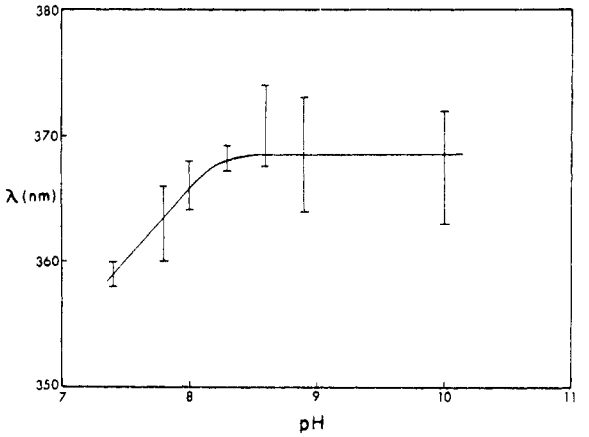


FIGURE 1: The pH dependence of the deuteroferriheme monomer-dimer isosbestic point at 25 °C (μ = 0.1). Buffer: HCO_3^-, CO_3^{2-} at pH ≥ 8; $H_2PO_4^-$, HPO_4^{2-} at pH < 8.

version, it is desirable to follow absorbancy changes at the wavelength of the heme monomer-dimer isosbestic so they will represent measures of intermediate formation uncomplicated by any accompanying changes in monomer-dimer distribution. Toward this end, an earlier investigation (Jones et al., 1974a) had been conducted at λ 377 nm (pH 7.75 and 8.5) based on previous observations, over a limited pH range, of deuteroferriheme absorption in aqueous detergent systems (Robson, 1973). Recent work by Jones and Mantle (1977, unpublished research), however, indicates this isosbestic to be at 357 nm in nondetergent media at pH 7.4, and this has prompted us to determine its pH dependence over the wider region to be employed for studies of intermediate formation in aqueous solution. The results are depicted in Figure 1, where absorbance data were obtained at total deuteroferriheme concentrations of 0.5, 1, and 5×10^{-5} M in 1.0-, 0.5-, and 0.1-cm cells. The variation of the isosbestic is greatest in phosphate buffer, diminishes with decreasing acidity, and becomes (within experimental error) independent of pH above 8.5, where the heme exists predominantly in dimeric form at all concentrations examined (Jones et al., 1974b). A pH dependence of the isosbestic is not surprising and, indeed, is anticipated in view of the known acidity of monomeric and dimeric heme whose respective pK_a values are 7.1 and 7.4 (Brown et al., 1970a).

Kinetics of Intermediate Formation. Data obtained from stopped-flow studies at different total (stoichiometric) concentrations of heme [T] and hydrogen peroxide at 25 °C over the range pH 8.69 to 10.08 are shown in Table I. Also listed are values of α, the fraction of deuteroheme existing as monomer in several buffered solutions as a function of [T] (Prudhoe, 1971; Jones et al., 1974b).

In the discussion to follow, these results are interpreted in terms of the model depicted in reaction 2, wherein I′ is treated as a kinetically significant entity which, along with I, is obtained in the steady state at t_∞. There is no implication as to the structure of I′ or even its singularity as a molecular species, although, again in a formal sense, it is treated as a mechanistic analogue of catalase compound I.[1] Recognizing $\alpha = [M]/[F]$, where [F] denotes the total concentration of free (uncomplexed) heme at any time, i.e., $[F] = [M] + 2[D] = [T] - [I] - [I']$ where [T] denotes the initial molar concentration of heme calculated as monomeric iron(III), one obtains from reaction 2

$$\frac{d[I]}{dt} = k_1\alpha([T] - [I'])[H_2O_2] - (k_1\alpha[H_2O_2] + k_2 + k_3)[I] \quad (3)$$

and

$$\frac{d[I']}{dt} = k_3[I] - k_4[I'][H_2O_2] \quad (4)$$

Assuming that a steady state in [I] is rapidly achieved, e.g., in the time of mixing, it follows that

$$[I]_{ss} = \frac{([T] - [I'])[H_2O_2]}{K_m/\alpha + [H_2O_2]} \quad (5)$$

whence

$$[I']_{ss} = \frac{k_3[T]}{k_3 + k_4(K_m/\alpha + [H_2O_2])} \quad (6)$$

where $K_m = (k_2 + k_3)/k_1$. Equation 4 may be rewritten

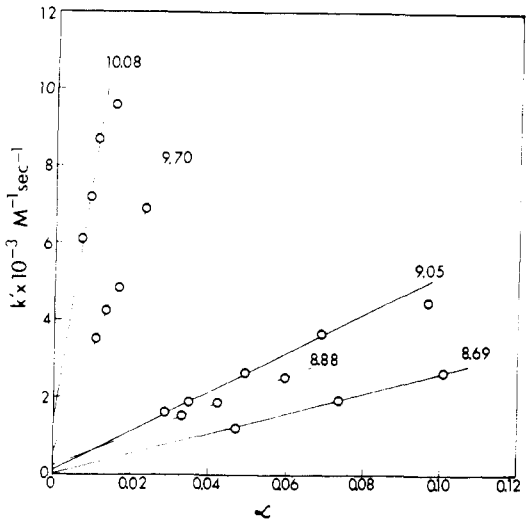


FIGURE 2: Variation of observed rate with degree of dissociation of deuteroferriheme dimer in HCO_3^-, CO_3^{2-} buffer; pH 8.69–10.08 at 25 °C (μ = 0.1). $k' \equiv k_{obsd}/[H_2O_2] = m\alpha + b$.

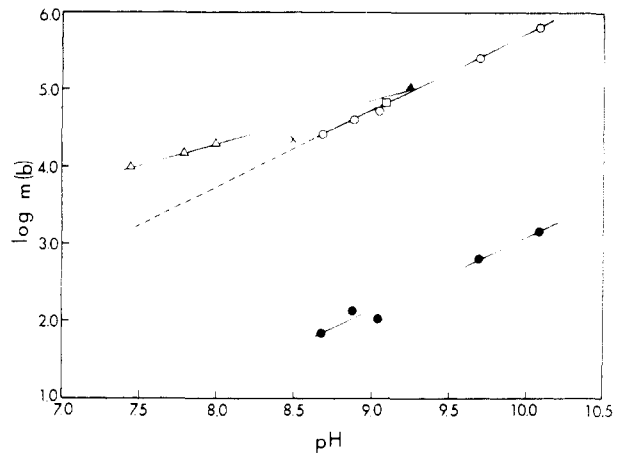


FIGURE 3: The pH and buffer concentration dependence of m and b (from $k' = m\alpha + b$) at 25 °C, μ = 0.1. For log m vs. pH: (○) $[HCO_3^-]$ + $[CO_3^{2-}]$ = 0.035 M; (△) $[H_2PO_4^-]$ + $[HPO_4^{2-}]$ = 0.033 M; (▲) 0.033 M HPO_4^{2-}; (□) 0.033 M $HAsO_4^{2-}$; (×) 0.017 M $C_6H_5O_7^{3-}$. For data in carbonate buffer, slope = 1.01; correlation coefficient = 0.999. For log b vs. pH: (●) $[HCO_3^-]$ + $[CO_3^{2-}]$ = 0.035 M, slope = 0.97; correlation coefficient = 0.985.

$$\frac{d[I']}{dt} = \frac{k_3[T][H_2O_2]}{K_m/\alpha + [H_2O_2]} - [I'][H_2O_2]\frac{k_3 + k_4(K_m/\alpha + [H_2O_2])}{K_m/\alpha + [H_2O_2]} \quad (7)$$

whereupon substitution for [T] in accordance with eq 6 yields on integration

$$\ln\left[\frac{[I']_{ss}}{[I']_{ss} - [I']}\right]_{[I']=0}^{[I']} = [H_2O_2]\frac{k_3 + k_4(K_m/\alpha + [H_2O_2])}{K_m/\alpha + [H_2O_2]} \quad (8)$$

Using previously described notations for molar extinction of species (Jones et al., 1974a), it can be shown that

$$\frac{A_t - A_\infty}{A_0 - A_\infty} = \frac{[I']_{ss} - [I']}{[I']_{ss}} \quad (9)$$

Thus, under pseudo-first-order conditions, i.e., where the initial H_2O_2 concentration $[H_2O_2]_0$, is in large excess of heme, the first-order rate constant, k_{obsd}, is determined from the plot of ln $(A_t - A_\infty)$ vs. t. In accordance with this scheme

$$k_{obsd} = \frac{k_3[H_2O_2]}{K_m/\alpha + [H_2O_2]} + k_4[H_2O_2] \quad (10)$$

Consistent with predictions of eq 10, it is found that k_{obsd} increases linearly with increasing $[H_2O_2]_0$ at the lower substrate concentrations with some curvature (decreasing positive slope) developing at higher concentrations. In this linear region, presumably $K_m/\alpha \gg [H_2O_2]$ and, defining $k' \equiv k_{obsd}/[H_2O_2]$, it follows that

$$k' \simeq \alpha\frac{k_3}{K_m} + k_4 \quad (11)$$

Values of k' obtained in this region are plotted vs. α in Figure 2.

The results offer a measure of consistency with eq 11 and, therefore, reaction 2 in the sense that a linear relationship of k' with α giving a nonzero intercept is indeed obtained. Both slope (m) and intercept (b) are clearly pH dependent,[2] the linear (logarithmic) dependence of each with near unit slope being depicted in Figure 3. It is evident that in terms of eq 11 the reaction of the peroxidatic intermediate with H_2O_2 (reflected in the k_4 term) will represent a proportionately greater contribution to k_{obsd} under conditions where α is relatively small, such as at high heme concentrations and high pH. Since our previous kinetic investigation (Jones et al., 1974a) was restricted to pH 7.75 and 8.5, it is not surprising that such a term was not previously detected.

Although it seems possible that the first-order dependence of rate on $[OH^-]$ may be due to acid dissociation of some function in the heme molecule, e.g., a ligand water molecule bound weakly to the Fe(III) center (such ionization has been proposed by Brown et al., 1970a), a more reasonable explanation may be one involving ionization of substrate to the perhydroxyl anion, with HO_2^- being the active-substrate species in two separate steps embodied in reaction 2: (a) the formation of I, as reflected in attack via k_1 on heme monomer [note $k_3/K_m = k_1k_3/(k_2 + k_3)$], and (b) the peroxidatic action of I′ as reflected in the k_4 term. This suggestion seems attractive in view of the known pH dependence of rates of decomposition of hydrogen peroxide by various iron(III)-centered catalysts, where an inverse first-order dependence on $[H^+]$ has been demonstrated for monomeric proto- and deuteroferriheme, and where activities in the region pH ≥ 11 approach those exhibited by bovine and bacterial catalase whose rates are pH independent over the range 6–10.5 (Brown et al., 1970b).

Assuming pK_a = 11.68 for H_2O_2 at 20 °C and μ (ionic strength) = 0.01 (Evans and Uri, 1949) and taking 8.9 kcal/mol for the enthalpy of acid dissociation (Sager and Hoffsommer, 1969), one estimates pK_a = 11.5 at 25 °C and μ = 0.1.[3] Defining $(k)_b = k(H_2O_2)/(HO_2^-) = k[H^+]/K_a$, one obtains the following kinetic parameters in accordance with reaction 2, assuming HO_2^- as substrate: $(k_3/K_m)_b = 1.7 \times 10^7$ M^{-1} s^{-1} and $(k_4)_b = 6 \times 10^4$ M^{-1} s^{-1}. The value of $(k_3/K_m)_b$ is comparable to those second-order rate constants observed for the formation of peroxidatic intermediate from undissociated hydrogen peroxide and a number of heme enzymes, e.g.,

[2] The degree of uncertainty in the value of b increases with decreasing pH.

[3] Extrapolation of Debye–Huckel theory to μ = 0.1 is not totally warranted, and the pK_a value so obtained is an approximate one used only for the estimation of rate constants.

TABLE II: Variation of Rate with Concentrations of Acidic Buffer Component.[a]

	$[H_2PO_4^-]^b \times 10^3$ (M)	k_{obsd} (s^{-1})	$k' \times 10^{-3}$ (M^{-1} s^{-1})
pH 7.33	0.42	0.348	1.98
$[H_2O_2]_0 = 1.76 \times 10^{-4}$ M	0.56	0.374	2.13
	0.70	0.415	2.36
	0.98	0.431	2.45
	1.40	0.513	2.91
pH 7.43	2.10	0.413	2.85
$[H_2O_2]_0 = 1.45 \times 10^{-4}$ M	2.80	0.409	2.82
	3.50	0.393	2.71
	4.20	0.400	2.76
	4.90	0.424	2.92
	5.00	0.476	3.28
	6.30	0.482	3.32
	7.00	0.513	3.54
	$[H_2AsO_4^-]^b \times 10^3$ (M)	k_{obsd} (s^{-1})	$k' \times 10^{-3}$ (M^{-1} s^{-1})
pH 7.37	0.46	0.370	2.16
$[H_2O_2]_0 = 1.71 \times 10^{-4}$ M	0.57	0.401	2.35
	0.80	0.427	2.50
	1.15	0.470	2.75
	2.29	0.521	3.05
pH 7.48	3.44	0.462	3.12
$[H_2O_2]_0 = 1.48 \times 10^{-4}$ M	4.00	0.460	3.11
	4.59	0.468	3.16
	5.16	0.481	3.25
	5.73	0.515	3.48
	$[HC_6H_5O_7^{2-}]^b \times 10^4$ (M)	k_{obsd} (s^{-1})	$k' \times 10^{-3}$ (M^{-1} s^{-1})
pH 7.4	1.58	0.366	2.08
$[H_2O_2]_0 = 1.76 \times 10^{-4}$ M	1.98	0.405	2.30
	2.37	0.428	2.43
	3.17	0.505	2.87
	3.56	0.536	3.05
	3.96	0.565	3.21

[a] pK_a values (Sillen and Martell, 1964): $H_2PO_4^-$, 7.21; $H_2AsO_4^-$, 6.97; $HC_6H_5O_7^{2-}$, 6.39 ([T] = 2.5×10^{-6} M; t = 25 °C; μ = 0.1; λ 359.5 nm). [b] Acid component/total buffer at pH 7.4: phosphate, 0.2; arsenate, 0.16, citrate, 0.014.

horseradish peroxidase (2.00×10^7 M^{-1} s^{-1}; Davies et al., 1976), cytochrome *c* peroxidase (4.7×10^7 M^{-1} s^{-1}; Loo and Erman, 1975), bacterial catalase (1.55×10^7 M^{-1} s^{-1}), and horse liver catalase (4.40×10^6 M^{-1} s^{-1}; Kremer, 1975).[4]

It is worthwhile to further compare values of k_3/K_m and k_4 in terms of what is implied through reaction 2 regarding the fractional conversion of total heme to I′ in the steady state. From eq 6, this is indicated to be

$$\frac{[I']_{ss}}{[T]} = \left[\frac{\alpha k_3}{K_m}\right] \Big/ \left[\frac{\alpha k_3}{K_m} + k_4\right] \qquad (12)$$

for $K_m/\alpha \gg [H_2O_2]$. The values given in Table I show this fraction to vary from about 0.7 at pH 10.1 to >0.9 at pH 8.7, and, although single values of α are employed in such calculations, in most cases, this may not be unjustified. Thus, for a study in which 80% conversion of heme to I′ has occurred at t_∞, and assuming $[I]_{ss}$ to be very small, the total change in α over a period of two half-lives would be only that corresponding to a reduction of free (uncomplexed) heme from [T] to 0.4 [T]. Under such conditions, a large departure from linear first-order kinetics is not expected. More extensive conversion is predicted at pH < 8.7; however, phosphate buffer solutions are employed for studies in this region and anomalous effects in such solutions have been noted both with regard to heme dissociation (Prudhoe, 1971) and heme activity (Jones et al., 1973a). Hence, there is probably less justification in applying eq 12 to such data. Indeed, in view of the results obtained with particular buffer components described below it is clear that further modification of reaction 2 is necessary for such systems.

Kinetic Effects of Selected Buffer Systems. Kinetic data on "intermediate" formation as a function of phosphate, arsenate, and citrate buffer concentration are given in Tables II and III and Figures 3 and 4. The variation in observed rate with concentration of the conjugate acid in a given buffer pair (Figure 4) is more pronounced at the lower concentrations with plateau regions approached for $[H_2PO_4^-]$ or $[H_2AsO_4^-]$ > 0.003 M at pH 7.4 (μ = 0.1). Limited heme solubility in solutions of higher ionic strength restricts the extent of study of the concentration dependence of the monohydrogen citrate ion.

That rate enhancement is due to an effect of the *acid* component of a given buffer pair, e.g., H_2A^{-n} of the H_2A^{-n}, $HA^{-(n+1)}$ system, is further depicted in Figure 3, which shows the variation of rate with pH for a given total phosphate buffer concentration of 0.033 M. Log m (the slope of a plot of k' vs. α) vs. pH is linear with positive slope of smaller magnitude than that (unit slope) which depicts the pH dependence as determined in HCO_3^-, CO_3^{2-} buffer. Significant also is the fact that in 0.033 M HPO_4^{2-} (no added $H_2PO_4^-$, pH 9.25) the rate is that which is predicted from the pH profile alone. Comparable results are obtained with 0.033 M $HAsO_4^{2-}$ (no added

[4] Catalase values are calculated per heme subunit.

TABLE III: Kinetic Parameters Obtained in Selected Buffer Systems at 25 °C (μ = 0.1).

pH	λ (nm)	[T] × 10^5 (M)	α[a]	k' × 10^{-3} (M^{-1} s^{-1})[b]	m × 10^{-5} [c]	log m
8.00[d]	366	0.20	0.235	4.55	0.201	4.30
		0.40	0.173	3.20		
		0.80	0.126	2.37		
7.79[d]	363.5	0.125	0.349	5.12	0.149	4.17
		0.25	0.262	3.97		
		0.50	0.194	2.89		
		1.0	0.142	2.04		
7.45[d]	359.5	0.20	0.394	3.65	0.0984	3.99
		0.40	0.299	2.68		
		0.80	0.223	1.97		
9.25[e]	368.5	0.50	0.0394	4.32	1.10	5.04
9.09[f]	368.5	0.50	0.0477	3.12	0.654	4.82
8.49[g]	368.5	0.50	0.0929	2.17	0.234	4.37

[a] From Prudhoe (1971). [b] $k' = k_{obsd}/[H_2O_2]$. [c] From $k' = m\alpha + b$; $b \simeq 0$. [d] $[Na_2HPO_4] + [KH_2PO_4] = 0.033$ M. [e] 0.033 M HPO_4^{2-}. [f] 0.033 M $HAsO_4^{2-}$. [g] 0.017 M $C_6H_5O_7^{3-}$.

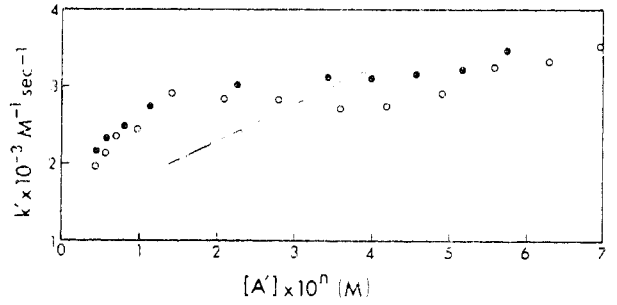
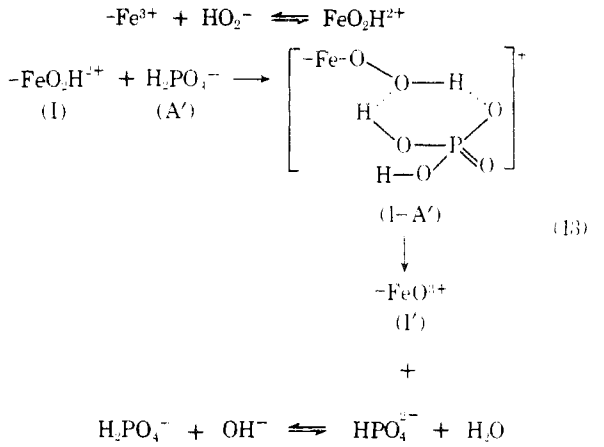


FIGURE 4: Variation of observed rate with concentration of acidic buffer component, A', at 25 °C and pH 7.4 (μ = 0.1). (○) $H_2PO_4^-$, (n = 3); (●) $H_2AsO_4^-$, (n = 3); (□) $HC_6H_5O_7^{2-}$ (n = 4). $k' = k_{obsd}/[H_2O_2]$.

$H_2AsO_4^-$) and 0.017 M citrate, $C_6H_5O_7^{3-}$ (no added $HC_6H_5O_7^{2-}$). At lower pH, then, the vertical distance between a point on this line and one on the line obtained by extrapolation, toward lower pH, of the data obtained in carbonate buffer presumably measures an effect of the acidic buffer component (in this case $H_2PO_4^-$) over that due to water and solvated hydrogen ion. Thus, the basic component of a given buffer pair has a negligible effect on the rate, except as it helps to establish the pH of the solution. Such data strongly suggest that "intermediate" formation from deuteroferriheme and H_2O_2 is subject to *general acid catalysis*. Added qualitative support is provided by the fact that, for the three systems thus far described, the kinetic effect of a given acid appears to increase with increasing acidity.

Although it is evident that the system is not sufficiently understood to permit a definitive statement of the origin of such buffer effects, it is interesting to consider reaction 2 as a starting point for speculation. If, in accordance with eq 11, the effect of $H_2PO_4^-$ is considered in terms of an increase in the apparent value of k_3, a general acid-catalyzed conversion of I to I' may be suggested (tentatively depicted as $I \xrightarrow{k_3'A'} I'$ where A' denotes the acidic species). Previous speculation on the nature of the peroxidatic intermediate originating in various iron(III) systems includes a "ferryl"-type structure containing the Fe-O linkage (Moss et al., 1969; Hager et al., 1972; Schonbaum and Lo, 1972; Cotton et al., 1973; Aasa et al., 1975). In an appropriate oxidase enzyme, the formation of such a species could be imagined to occur via dehydration of a primary enzyme-substrate complex. An analogous conversion of I to I' in the heme system, in which HO_2^- is viewed as the substrate, would involve extraction of OH^- from an iron(III)-HO_2^- complex, a process in which catalysis by selected Brönsted acids might be expected. A simplified view of such a process involving $H_2PO_4^-$ is tentatively depicted in eq 13. The bracketed species in eq 13 might be regarded as an *activated* complex arising through *concerted* attack or, alternatively, as an *intermediate* complex of unspecified lifetime originating through (possibly reversible) attack of acid on I, perhaps via hydrogen bonding. In the former case, k_3' would denote a single bimolecular rate constant and in the latter, a composite of an equilibrium constant for I-A' formation and a rate constant for conversion of I-A' to I'. General acid catalysis is a consequence of either model. Of these alternatives, the reversible formation of a hydrogen-bonded I-A' complex would appear to be favored on the basis of the apparent saturation effect on rate of $H_2PO_4^-$ and $H_2AsO_4^-$ concentration. Such discussion probably reflects considerable oversimplification, however, and will remain highly conjectural until more definitive studies have been made on the nature and extent of acid catalysis.



(13)

It should be noted, also, that reexamination of the mechanism of reaction 2 may be necessary in view of recent studies of the "stoichiometry" of the reaction of deuteroheme with selected substituted perbenzoic acids. From spectral studies, it appears that these pseudosubstrates react quantitatively in a mole ratio peracid to heme of 1:2 (Jones et al., unpublished results). It is possible that this represents complexation of a peroxidatically active intermediate and a second unit of heme, which if also occurring in H_2O_2 solution, e.g., involving I', must be accommodated in any model which attempts to portray the stoichiometric mechanism of "intermediate" formation. Thus,

what has been depicted as step 4 in reaction 2 may actually represent a composite of peroxidatic reactions involving both I′ and a heme derivative of I′ or some other heme-peroxo-substrate complex. Further study of peroxobenzoic acid-heme systems, including the peroxidatic activity of their resulting solutions, is in progress (Jones and Mantle, 1977, unpublished results).

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