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Oxidation of Deuteroferrihaem by Hydrogen Peroxide

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1. The oxidation of deuteroferrihaem by H_2O_2 to bile pigment and CO was studied both by stopped-flow kinetic spectrophotometry and mass spectrometry, at 25°C, $I = 0.1$ M.
2. Spectrophotometric studies imply that, at constant pH, the rate of bile pigment formation is first-order with respect to $[H_2O_2]$ and also proportional to [deuteroferrihaem monomer]. The effect of pH on the apparent second-order rate constant suggests that acid-ionization of deuteroferrihaem monomer is important in the reaction mechanism.
3. The relative rates of formation of O_2 (from catalytic decomposition of H_2O_2) and CO (from oxidation of ferrihaem) have been measured by mass spectrometry. The results are in excellent agreement with those obtained by combining kinetic data for catalytic decomposition (Jones *et al.*, 1973, preceding paper) with the spectrophotometric results for deuteroferrihaem oxidation.

Oxidative cleavage of the porphyrin moiety accompanies and complicates studies of the catalytic decomposition of H_2O_2 by ferrihaems, and these reactions have received considerable attention because of their relationship to the natural degradation of haemoproteins (see Falk, 1964). In both processes a methene bridge carbon atom is eliminated, the reaction products being CO and a bile pigment, such as biliverdin. At adequate peroxide concentrations further oxidation of bile pigment may take place, forming colourless dipyrrollic fragments. A stoicheiometric relationship has been demonstrated between the amount of CO exhaled and the amount of haemoglobin broken down per day by human subjects. The formation *in vitro* of bile pigment and CO in stoicheiometric proportions has been demonstrated from myoglobin (Sjöstrand, 1952), haemoglobin (Anan & Mason, 1961) and protoferrihaem (haemin) (Ludwig *et al.*, 1957).

Brown & Jones (1968) studied the kinetics of the oxidation of protoferrihaem to bile pigment by H_2O_2 by using a conventional spectrophotometric method. In the present work we have examined the oxidation of deuteroferrihaem by using stopped-flow spectrophotometry. Recent studies of the catalase activity of ferrihaems (Brown *et al.*, 1970*a*; Jones *et al.*, 1973) have revealed the importance of ferrihaem dimerization (Brown *et al.*, 1970*b*; Prudhoe, 1971) in the reaction. In the accessible range of deuteroferrihaem concentrations the degree of dimerization may be varied widely and permits an examination of the role of dimerization in the ligand oxidation reaction. A second series of experiments making use of a mass-spectrometric method have yielded results that are in excellent agreement with the kinetic data obtained spectrophotometrically and catalytic data.

Experimental and Results

General methods

The specification and methods of assay of materials have been described previously (Brown *et al.*, 1970*b*; Jones *et al.*, 1973). Deuteroferrihaem solutions were prepared by method A (Brown *et al.*, 1970*c*). Kinetic spectrophotometric studies were made at 25°C, $I = 0.1$ (added electrolyte NaCl) in a Durrum–Gibson D-110 stopped-flow spectrophotometer. Mass-spectrometric measurements were made on an AEI MS3 mass spectrometer.

Stopped-flow experiments

The admixture of deuteroferrihaem solution with H_2O_2 results in several distinct changes in the u.v./visible absorption spectrum of the solution. A complex deuteroferrihaem–H_2O_2 intermediate is rapidly formed, with a substantial decrease in extinction in the Soret band region (Portsmouth & Beal, 1971; Prudhoe, 1971). This species has peroxidatic properties and is involved in catalatic action. There is kinetic evidence for the reversible formation of a precursor complex (Michaelis complex) before the peroxidatic intermediate. Studies of the effect of changing ferrihaem concentration on the kinetics of formation of the peroxidatic intermediate imply that it derives from the reaction of H_2O_2 (or HO_2^-) with deuteroferrihaem monomer (Prudhoe, 1971).

Events subsequent to the formation of the intermediate depend upon the $[H_2O_2]$ employed. At an adequately low $[H_2O_2]$ the extinction of the solution is almost completely restored. At higher $[H_2O_2]$ oxidation of the porphyrin ligand to bile pigment becomes increasingly important until no restoration

of the Soret band extinction is observed and the extinction decreases irreversibly with time.

The ligand oxidation reaction is most conveniently studied at wavelengths in the visible range of the spectrum, where formation of the peroxidatic intermediate results in a small, rapid increase in extinction and oxidation is observed as a subsequent large decrease in extinction. Measurements were made at relatively high $[H_2O_2]$, so that initial-rates analysis could be employed. Most of our experiments were carried out at 629nm, which is an isosbestic point for the deuteroferrihaem monomer–dimer system. (Some experiments were performed at 545nm.) Under these conditions, if the extinction per unit path-length of the ferrihaem solution in the initial steady state for the peroxidatic intermediate is

$$E^0 = \epsilon_F[T] \quad (1)$$

after reaction time t:

$$E^t = \epsilon_F[F] + \epsilon_B[B] \quad (2)$$

where [T] is the initial (stoicheiometric) ferrihaem concentration, [F] is the remaining concentration of ferrihaem species, [B] the concentration of bile pigment formed and ϵ_F and ϵ_B are the extinction coefficients of ferrihaem and bile pigment respectively.

Since $[T] = [F] + [B]$, and by introducing $\Delta E = E^0 - E^t$, we can express the rate of reaction as:

$$V_i = \frac{d[F]}{dt} = \frac{d[B]}{dt} = \frac{d\Delta E}{dt}\,\frac{1}{(\epsilon_F - \epsilon_B)} \quad (3)$$

The stopped-flow spectrophotometer was calibrated by filling the syringes with de-gassed ferrihaem solution and distilled water and setting the 100% transmission datum by using the ferrihaem–water (1:1) solution. The water syringe was then filled with de-gassed H_2O_2 solution and the reaction was initiated. The sensitivity was adjusted so that the initial linear part of the oxidation reaction trace filled the screen. A number of experiments were then carried out under the same conditions but with different time-scales, to test reproducibility and to enable extrapolation to the time of mixing. The set of traces was stored on the oscilloscope and then photographed with a Polaroid camera; a typical set is illustrated in Fig. 1(*a*). Under the conditions of our experiments further oxidation of bile pigment was not significant and good infinity values were obtained by changing the range setting of the spectrophotometer and re-triggering the time base at the end of the reaction (Fig. 1*b*).

Fig. 2 shows that the reaction is accurately first order with respect to $[H_2O_2]$, under a variety of conditions and up to 0.2M-H_2O_2. In the analysis of the results of subsequent experiments (at $[H_2O_2] =$ 0.4M) a first-order dependence of rate on $[H_2O_2]$ was

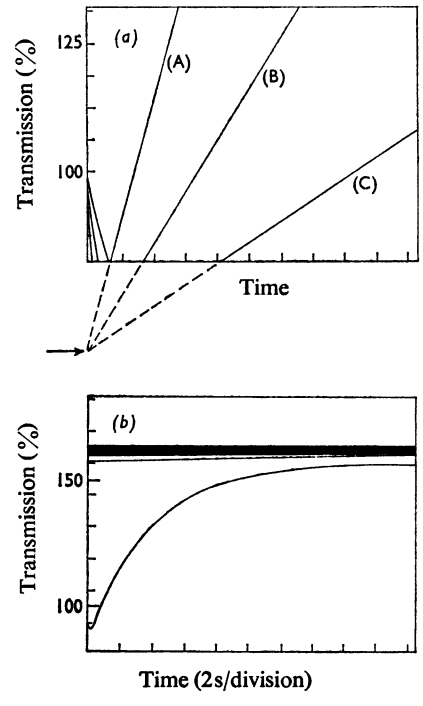


Fig. 1. *Typical stopped-flow traces*

(*a*) The traces show the initial rate of deuteroferrihaem oxidation (under the conditions: [deuteroferrihaem] = 200μM; $[H_2O_2] = 0.4$M; pH7.75; temperature = 25°C) as measured by the increase in % transmission at $\lambda = 629$nm. The time-scales in the three experiments were (A) 1.0, (B) 0.5 and (C) 0.2s/division. The broken lines indicate extrapolation to the initial steady state for the peroxidatic intermediate (arrow). (*b*) An experiment under similar conditions but with decreased spectrophotometric sensitivity and repeated re-triggering of the time-base for determination of the infinity value.

assumed. In these experiments the variation of the pseudo-first-order rate constant $V_i/[H_2O_2]$ was examined over a range of deuteroferrihaem concentrations in the pH range 6.95–9.50 (Na_2HPO_4–KH_2PO_4 buffers and $NaHCO_3$–Na_2CO_3 buffers). Fig. 3 illustrates the pH-dependence of $(\epsilon_F - \epsilon_B)$. At constant pH, $V_i/[H_2O_2]$ did not show a simple dependence on [T]. To test for a possible role of ferrihaem dimerization in the reaction, plots of $V_i/[H_2O_2]$ against α were constructed. [α is the fraction of ferrihaem present as monomer (Brown *et al.*, 1970*a*) and values were calculated from the most recent value of the dimerization constant: $K = K_{obs.}[H^+] = 3.4 \times 10^{-2}$ (Prudhoe, 1971).] These were straight lines, which passed through the origin within the experi-

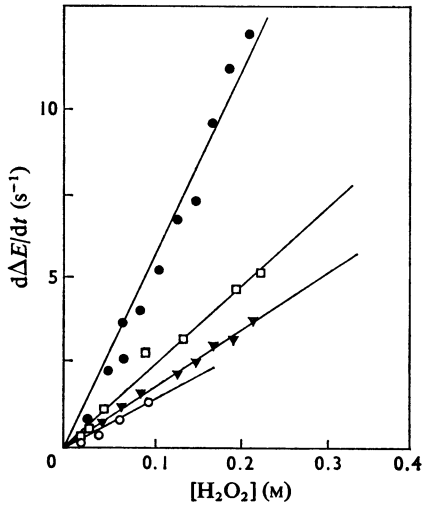


Fig. 2. *First-order dependence on* $[H_2O_2]$ *of the rate of oxidation of deuteroferrihaem (expressed as* $\Delta E/dt$ *at 545 nm)*

The experiments were carried out at 25°C, pH7.47, $I = 0.1$ (Na_2HPO_4–KH_2PO_4 buffer). Deuteroferrihaem concentrations (μM): ●, 128; □, 47.5; ▼, 32.0; ○, 20.2.

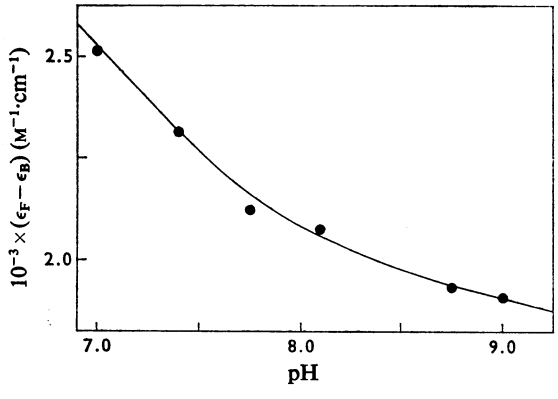


Fig. 3. *pH-dependence of* $(\epsilon_F - \epsilon_B)$ *at 629 nm*

ϵ_F is the extinction coefficient of ferrihaem solutions in the initial steady state for the peroxidatic intermediate. ϵ_B is the extinction coefficient of the bile pigment formed in the oxidation reaction.

mental error. This result suggests that the appropriate rate law for the reaction is:

$$V_i = k_2[M][H_2O_2] \quad (4)$$

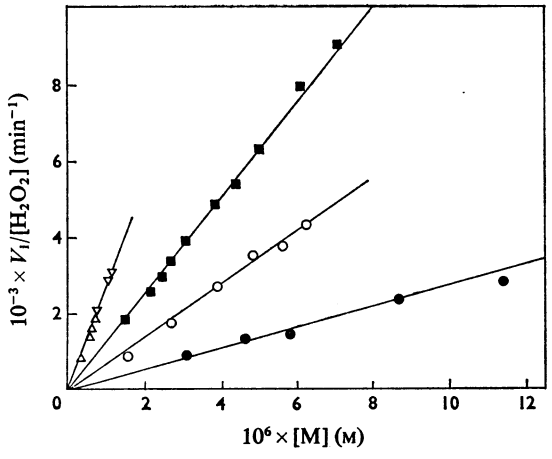


Fig. 4. *Dependence of* $V_i/[H_2O_2]$ *(measured at 629 nm) on the concentration of deuteroferrihaem monomer* ($[M]$)

▽, pH9.50; △, pH9.00 ($NaHCO_3$–Na_2CO_3 buffers). ■, pH7.75; ○, pH7.40; ●, pH6.95 (Na_2HPO_4–KH_2PO_4 buffers).

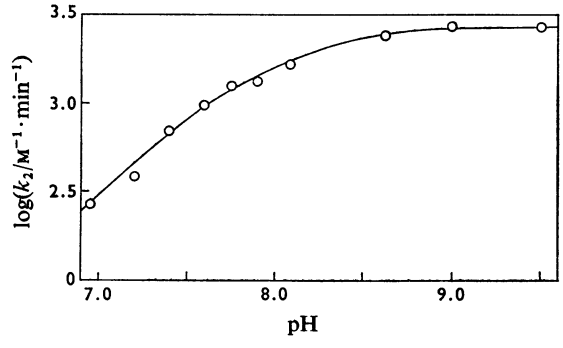


Fig. 5. *pH-dependence of* k_2

k_2 is the apparent second-order rate constant for the oxidation of deuteroferrihaem monomer by H_2O_2.

where M denotes deuteroferrihaem monomer and the second-order rate constant k_2 depends on $[H^+]$. The results are plotted according to this form in Fig. 4, and the pH-dependence of the derived values of k_2 in Fig. 5.

Mass-spectrometric measurements

An independent test of the spectrophotometric method described above was made by using a mass-spectrometric procedure. The basis of this procedure is as follows:

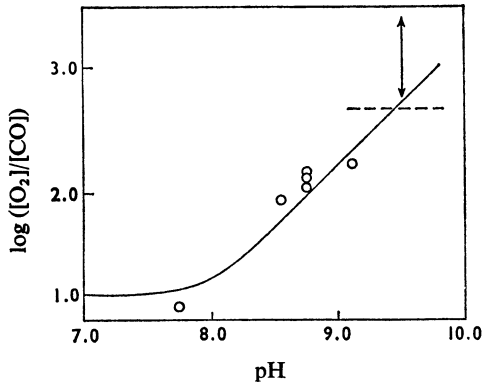


Fig. 6. *pH-dependence of* $[O_2]/[CO]$ *in the product gas obtained during the initial stages of reaction of deuteroferrihaem with* H_2O_2

The continuous line represents a''_M/k_2: a''_M was obtained from Jones *et al.* (1973), k_2 from Fig. 5. The horizontal broken line represents the sensitivity limit of the mass-spectrometric analysis ($[O_2]/[CO] = 500:1$). The arrow represents an experiment at pH 9.5 in which no CO was detected in the product gas.

For the catalytic decomposition of H_2O_2 by deuteroferrihaem, it has been established (Jones *et al.*, 1973) that the rate law (at adequately low $[H_2O_2]$, 4mM) is:

$$-\tfrac{1}{2}d[H_2O_2]/dt = d[O_2]/dt = a''_M[M][H_2O_2] \quad (5)$$

where the catalytic constant a''_M is proportional to $1/[H^+]$.

For the oxidation of deuteroferrihaem, the results described in the previous section show

$$\frac{d[B]}{dt} = \frac{d[CO]}{dt} = k_2[M][H_2O_2] \quad (6)$$

Thus, for small extents of reaction, the ratio $[O_2]/[CO]$ in the product gas should be related to the catalytic constant and the rate constant for oxidation according to:

$$\frac{[O_2]}{[CO]} = \frac{a''_M}{k_2} \quad (7)$$

Since independent measurements of a''_M and k_2 are available, a test of consistency may be obtained by measuring $[O_2]/[CO]$ in the product gas in the initial stages of reaction.

Samples (5ml) of 0.1mM-deuteroferrihaem in appropriate buffer and 5ml of 8mM-H_2O_2 were degassed in separate compartments of a reaction vessel attached to a vacuum line. The solutions were mixed under vacuum and allowed to react for 30s at 25°C. The gas evolved was taken into a sample vessel for mass-spectrometric analysis. The data were evaluated by using standard cracking-pattern correction procedures and established sensitivity factors. In particular the contribution of CO was evaluated by comparison of the (*m/e*) 14 peak height ($[^{12}C^{16}O]^{2+} + {}^{14}N$) and the (*m/e*) 29 peak height ($^{13}C^{16}O + {}^{14}N^{15}N$). The result was checked by comparison of $(CO + N_2)$ with the observed peak at (*m/e*) 28 and by calculating the contribution of N_2 from the observed argon (*m/e*) 40 peak.

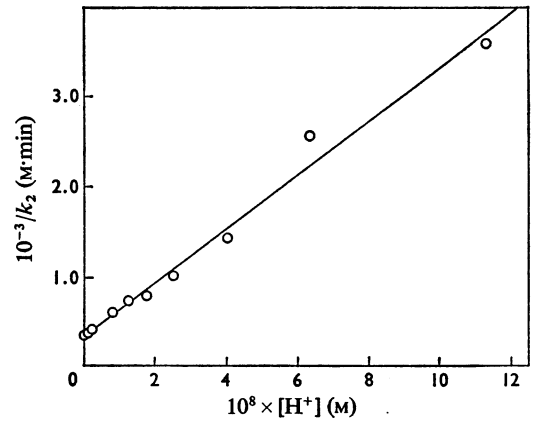


Fig. 7. *Relationship between* $1/k_2$ *and* $[H^+]$

Comparison of the data with the predictions of the mechanisms shown in Scheme 1 and eqns. (8) and (9).

The results are shown in Fig. 6. The continuous line represents the pH-dependence of a''_M/k_2 computed from the data of Jones *et al.* (1973) and the spectrophotometric data of the previous section. The broken line indicates the estimated limit of sensitivity of the mass-spectrometric method ($[O_2]/[CO] = 500:1$). The three independent sets of data are clearly internally consistent.

Discussion

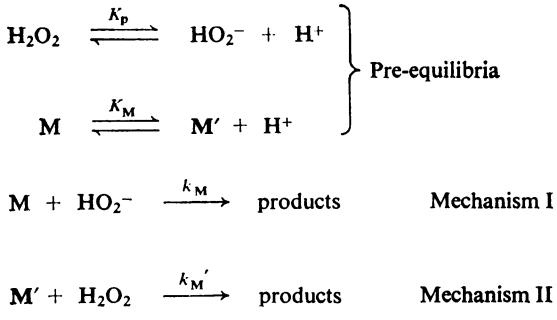
The variation of k_2 with pH suggests that ionization of deuteroferrihaem monomer (Brown *et al.*, 1970*a*) is relevant in the reaction mechanism. Two models are consistent with the form of the results and cannot be distinguished with the currently available data (Scheme 1).

According to mechanism I (Scheme 1), k_2 has the form:

$$k_2 = \frac{k_M K_p}{[H^+] + K_M} \quad \text{(provided that } [H^+] \gg K_p) \quad (8)$$

According to mechanism II

$$k_2 = \frac{k_M' K_M}{[H^+] + K_M} \quad (9)$$



Scheme 1. *Two models (see the Discussion section)*

A mechanism involving simultaneous contributions via both pathways is also possible. In all three cases a linear plot of $(1/k_2)$ against $[H^+]$ is predicted and this form describes the data very satisfactorily (Fig. 7). From Fig. 7, $pK_M = 7.9$ is calculated, which compares with the value of 7.1 reported by Brown *et al.* (1970*b*). The latter value was obtained from pH-dependence of the extrapolated values at infinite dilution of the extinction coefficients of deuteroferrihaem solutions and the result is therefore of low precision. Thus the rather modest measure of agreement between the two values of K_M may be reasonable, if not entirely satisfactory. Further experiments at very high pH would be valuable.

A more clear-cut result is provided by the data in the inferred protective influence of dimerization upon the sensitivity to oxidation of the porphyrin ring. This result is of interest in relation to the similar protection which the protein environment provides in haemoproteins. In this connexion it is noteworthy that protoferrihaem, which is much more highly dimerized than the deutero species under similar conditions, is oxidized much more slowly (by a factor of 10^3) and the effects of pH on the reaction are very similar (Brown & Jones, 1968).

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