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The Role of Peroxide in Haem Degradation

A STUDY OF THE OXIDATION OF FERRIHAEMS BY HYDROGEN PEROXIDE

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The oxidation of ferrihaems by H_2O_2 was studied as a model for haem catabolism. Rates of ferrihaem oxidation were evaluated by using a new computer-based method that measures the loss in catalytic activity of the ferrihaem during oxidation. For protoferrihaem, deuteroferrihaem, coproferrihaem and mesoferrihaem, oxidation proceeded via the monomeric species and no dimer contribution was detectable. The pH-dependence of oxidation was studied in the range 6.5–11. Within experimental error, the data were compatible with an inverse linear dependence on $[H^+]$. This was interpreted in terms of attack by HO_2^- on monomeric ferrihaem. The specific second-order rate constants for oxidation of monomeric species by HO_2^- were of the same order of magnitude for all the ferrihaems, and were in the sequence coproferrihaem> protoferrihaem> mesoferrihaem $\simeq$ deuteroferrihaem. A model is suggested involving formation of a ferrihaem monomer–peroxide complex, which may either dissociate with the formation of a peroxidatic intermediate or be involved in an intramolecular oxidation of the ferrihaem. Haem catabolism may occur via the same or a similar intermediate.

The oxidative degradation of haem to bile pigment (Scheme 1*a*, 1*b*) represents the major pathway by which higher animals are able to eliminate their unwanted haem. The relevant literature has been extensively reviewed (Lathe, 1972; Jackson, 1974; O'Carra, 1975; Schmid & McDonagh, 1975). A similar reaction is thought to be involved in the biosynthesis of the algal biliproteins, which act as accessory pigments in photosynthesis. In both animal and algal systems, haem cleavage requires molecular oxygen and involves elimination of a haem methene-bridge carbon atom as CO. ^{18}O-labelling studies have shown that the oxygen atom in CO and those incorporated into bile pigment (Scheme 1*b*) are derived from molecular oxygen (Tenhunen *et al.*, 1972; Brown & King, 1978; Troxler & Brown, 1977).

Although the initial step in haem catabolism almost certainly involves molecular oxygen, the precise way in which oxygen is activated is not clear. Brown (1976) has suggested that haem cleavage requires the formation of a haem–O_2 complex (as in oxyhaemoglobin or oxymyoglobin) and reduction of the bound oxygen, probably to peroxide. In this context, it is therefore of some importance to study the direct oxidation of haem derivatives by H_2O_2. Such degradation occurs readily in neutral or alkaline solution with the evolution of CO and the production of biliverdin (Gray, 1953). However, the product biliverdin is itself attacked by H_2O_2 to yield colourless dipyrrollic fragments known as propentdyopents (Scheme 1*c*; Gray, 1953). The net result of treatment of a solution of protoferrihaem with H_2O_2 is therefore a bleaching of the solution without accumulation of bile pigment. This reaction was studied spectrophotometrically by Brown & Jones (1968), who measured the kinetics of haem disappearance at 0°C. It was not possible to interpret these results in detail, however, because of problems involving haem aggregation (Falk, 1964). Subsequently, Brown *et al.* (1970) showed that between pH 6.5 and 11 aggregation of protoferrihaem and deuteroferrihaem (Scheme 1) is limited to dimerization according to the equation:

$$2M \underset{}{\overset{K}{\rightleftharpoons}} D + H^+$$

from which:

$$K = [D][H]^+/[M]^2 \qquad (1)$$

For protoferrihaem and deuteroferrihaem the values of K (which is dimensionless) were found to be 4.5 and 0.034 respectively in 0.033M-phosphate buffer (KH_2PO_4/Na_2HPO_4, I 0.1; Brown *et al.*, 1970; Jones *et al.*, 1974).

In a study using stopped-flow spectrophotometry, Jones *et al.* (1973) showed that the oxidation of deuteroferrihaem by H_2O_2 was much faster than oxidation of protoferrihaem under comparable conditions, consistent with the monomeric species being more reactive. By using the stopped-flow method, it was possible to measure the absorption coefficient of the immediate reaction product,

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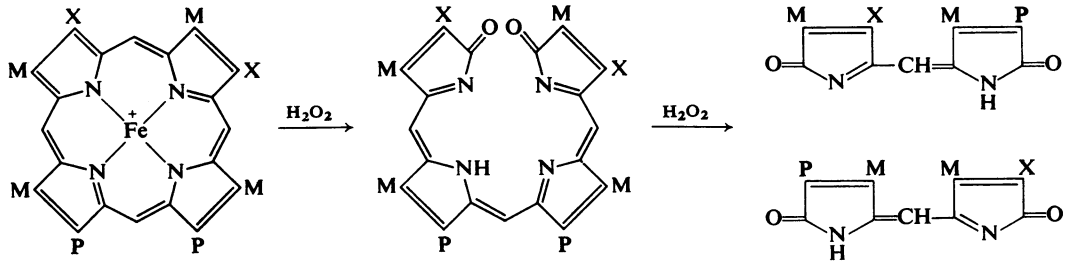


(*a*) Ferrihaem (*b*) Biliverdin (*c*) Propentdyopents

Scheme 1. *Degradation of ferrihaems by* H_2O_2

Protoferrihaem, X, $-CH=CH_2$; deuteroferrihaem, X, $-H$; mesoferrihaem, X, $-CH_2CH_3$; coproferrihaem, X, $-CH_2-CH_2CO_2H$.

assumed to be deuterobiliverdin. Comparable results were obtained by mass-spectrometric measurement of the CO produced. Analysis of these results showed that all of the oxidation reaction was carried by the monomeric species. The reaction was first order in H_2O_2 up to 0.2M-H_2O_2. In the present work a new computer-based method not requiring stopped-flow techniques is described for measurement of the kinetics of ferrihaem degradation by H_2O_2. The method has been applied to the oxidation of deuteroferrihaem, mesoferrihaem, coproferrihaem and protoferrihaem (Scheme 1), which have a wide range of dimerization constants, to further assess the importance of the monomer–dimer equilibrium and the mechanism of macrocyclic ring cleavage.

Experimental and Results

Materials and methods

All materials were as described in the previous paper (Hatzikonstantinou & Brown, 1978). Measurements of the catalytic decomposition of H_2O_2 by mesoferrihaem, coproferrihaem and deuteroferrihaem (except where stated below) were also as previously described and were continued until catalytic decomposition had ceased, i.e. to zero ferrihaem concentration. The initial value of $[H_2O_2]$ was 4mM in every case. Additional decomposition rate data were obtained from unpublished primary results for deuteroferrihaem (Dean, 1969) and protoferrihaem (S. B. Brown & P. W. Andrews, unpublished results). All data refer to 25°C and were obtained either in 33mM-phosphate buffer (KH_2PO_4/Na_2HPO_4 for the range pH6.5–8.3, Na_2HPO_4/NaOH for the range pH10.5–11) adjusted to I 0.1 with NaCl or 17mM-phosphate buffer adjusted to I0.05 with NaCl.

Determination of oxidation rate parameters

The rate of ferrihaem oxidation may be measured by following any property that changes on degradation. One such parameter is the catalytic activity of ferrihaems in the decomposition of H_2O_2. In the previous paper (Hatzikonstantinou & Brown, 1978), it was shown for deuteroferrihaem, mesoferrihaem and coproferrihaem that catalytic decomposition ceases before the concentration of H_2O_2 is decreased to zero, owing to oxidation of the catalyst. The rate equation for decomposition may be written:

$$-\tfrac{1}{2}\frac{d[H_2O_2]}{dt} = k_0[T][H_2O_2] \qquad (2)$$

where [T] represents the total ferrihaem concentration and k_0 is a second-order rate constant relating to decomposition. Because of catalyst oxidation, initial values of [T] are not maintained and the rate of decomposition becomes progressively less than that expected from eqn. (2). At any time, deviations from the rate law expressed in eqn. (2) are therefore related to the rate of catalyst oxidation given by:

$$-\frac{d[T]}{dt} = k_1[T][H_2O_2] \qquad (3)$$

where k_1 is a second-order rate constant relating to ferrihaem oxidation. Algebraic solution of eqns. (2) and (3) to yield values of k_0 and k_1 is not possible. The following numerical method was therefore used. The statistically best values of k_0 and k_1 were obtained by carrying out a numerical solution of the differential eqns. (2) and (3) with provisional values for the two rate constants. A method of the Runge–Kutta type (Mayers, 1962) was used with the values of $[H_2O_2]$ and [T] known at time zero. The differences between the values of $[H_2O_2]$ calculated at each time and the actual values were then converted into a sum of

squares and used in a minimization procedure of Powell (1968). The calculations were carried out on the ICL 1960A computer at the University of Leeds with a program written in ALGOL 60 and based on sub-routines taken from the Nottingham Algorithms Group Library. The computer output yielded values of k_1 and hence the initial rate of oxidation, R_i, corresponding to the conditions of each set of data.

Deuteroferrihaem

The computer-fit method for measurement of oxidation rates was initially applied to deuteroferrihaem oxidation, so that the method could be compared with known literature data (Jones *et al.*, 1973). The decomposition rate data used were those of the previous paper (Hatzikonstantinou & Brown, 1978) at I 0.05 and earlier work (Dean, 1969) at I 0.1. Fig. 1(*a*) shows plots of R_i versus [T] for the data at I 0.05 and constant initial 4mM-H_2O_2. It is clear that R_i does not vary linearly with [T], to be expected if monomeric and dimeric species behave differently in the oxidation reaction. Fig. 1(*b*) shows, however, that R_i varies linearly with [M], determined from the known value of the dimerization constant and eqn. (1). (For deuteroferrihaem at I 0.05, $K = 2.62 \times 10^{-2}$; Hatzikonstantinou, 1977.) Thus:

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

where k_2 is the second-order rate constant for oxidation of monomer, as found by Jones *et al.* (1973).

Similar relationships were obtained when the method was applied to the data at I 0.1 (Dean, 1969). For both sets of data, k_2 values were determined from the slopes of lines such as those in Fig. 1(*b*). Fig. 2 shows the pH dependence of k_2 plotted logarithmically. It is clear that the k_2 values show a distinct ionic strength dependence, those at I 0.05 being higher than those at I 0.1. The results of Jones *et al.* (1973) obtained by using the stopped flow method at I 0.1 are also shown in Fig. 2. It may be seen that the computer-fit method used in the present work yields data in close accord with these earlier results. This good agreement between the two completely different methods gives confidence in the application of the computer-fit method to the oxidation of other ferrihaems.

Coproferrihaem

The oxidation of coproferrihaem by H_2O_2 was studied by using decomposition rate data at I 0.05 (Hatzikonstantinou & Brown, 1978). Values of k_1 and hence R_i were obtained from the computer-fit

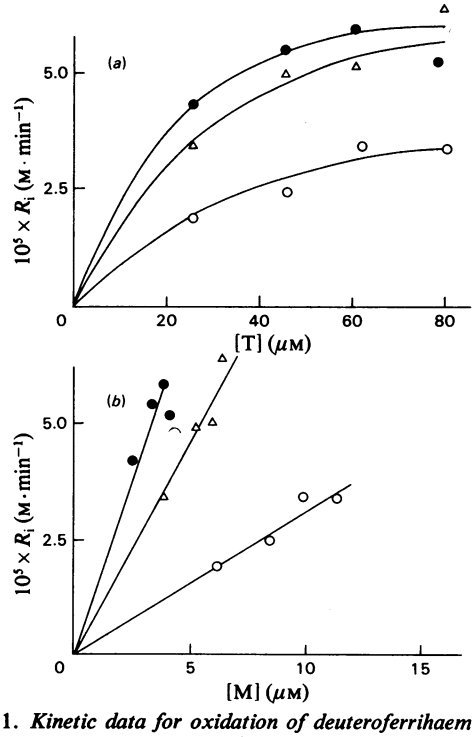


Fig. 1. *Kinetic data for oxidation of deuteroferrihaem by H_2O_2*

(*a*) Variation of the initial rate of ferrihaem oxidation (R_i) with total ferrihaem concentration ([T]). ●, pH7.86; △, pH7.45; ○, pH7.02. (*b*) Variation of R_i with monomer concentration ([M]), symbols as in (*a*) above. The values of [T] and [M] are initial values of these variables.

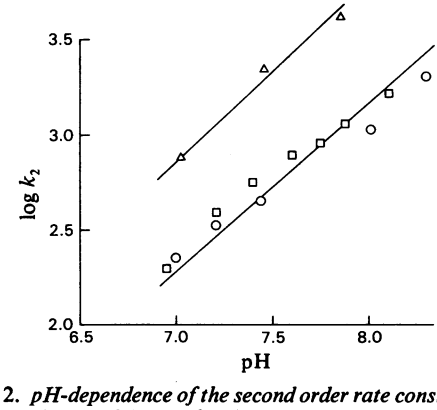


Fig. 2. *pH-dependence of the second order rate constant for oxidation of deuteroferrihaem monomer by H_2O_2*

△, Results from the present work at I 0.05; ○, results from the present work at I 0.1; □, results from Jones *et al.* (1973) at I 0.1. The lines have been drawn with a slope of unity (see the text).

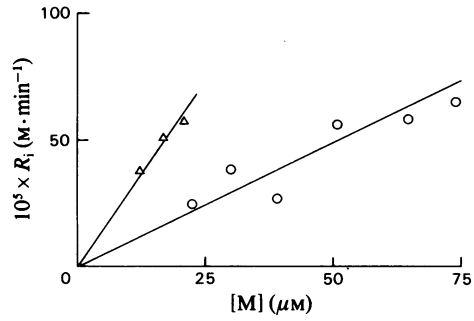


Fig. 3. *Kinetic data for oxidation of coproferrihaem by H_2O_2*
△, pH7.56; ○, pH7.81.

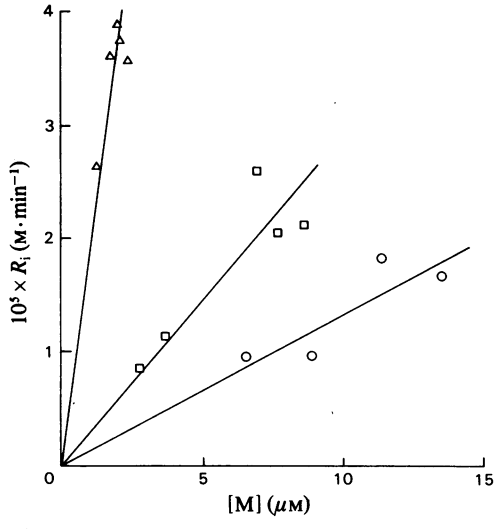


Fig. 4. *Kinetic data for oxidation of mesoferrihaem by H_2O_2*
△, pH8.11; □, pH7.02; ○, 6.62.

method described above for several initial values of [T] at each of four pH values. Calculations were restricted to pH values below 7.74 because of the very high rate of catalytic decomposition of H_2O_2 at higher pH (Hatzikonstantinou & Brown, 1978). Values of [M] were evaluated ($K = 1.76 \times 10^{-3}$; Hatzikonstantinou, 1977) and Fig. 3 shows the variation of R_i with [M] for coproferrihaem at selected pH values. Again there is considerable scatter, but the data are consistent with a linear relationship according to eqn. (4). Similar relationships were observed at other pH values studied. Values of k_2 were determined from the slopes of such graphs as described above.

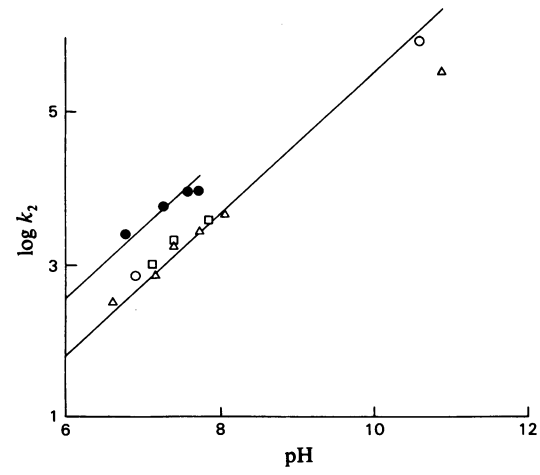


Fig. 5. *pH-dependence of rate constants for oxidation of various ferrihaems by H_2O_2*
●, Coproferrihaem (*I* 0.05); △, mesoferrihaem (*I* 0.05); □, deuteroferrihaem (*I* 0.05); ○, protoferrihaem (*I* 0.1). The lines have been drawn with a slope of unity (see the text).

Mesoferrihaem

The decomposition rate data for mesoferrihaem at *I* 0.05 (Hatzikonstantinou & Brown, 1978) were treated as described for coproferrihaem. Fig. 4 shows plots of R_i against [M] ($K = 5.47 \times 10^{-2}$; Hatzikonstantinou, 1977) at selected pH values which show a linear relationship consistent with eqn. (4) in spite of considerable scatter. Values of k_2 were determined from the slopes of these plots as before.

Protoferrihaem

For protoferrihaem ($K = 4.5$ at *I* 0.1), the proportion of monomer in solution is very small (about 0.1–0.01%). Only a limited amount of data was available (S. B. Brown & P. Andrews, unpublished work). However, this was used in the computer-fit method and values of k_2, determined as before, are shown in Fig. 5.

Discussion

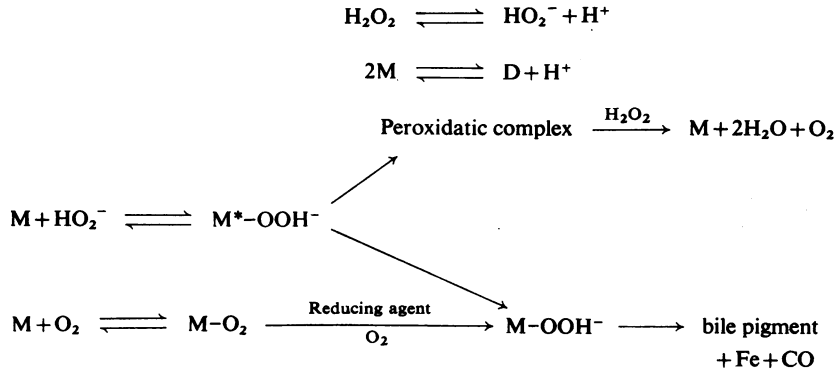
Values of k_2 (plotted logarithmically) are shown as a function of pH for all the ferrihaems studied in this work in Fig. 5. The solid lines have been drawn with a slope of unity. Although the results at high pH are limited, the data are consistent with an inverse linear dependence of k_2 on [H^+] at least up to pH 10. The results of the stopped-flow study by Jones *et al.* (1973) obtained in phosphate buffers (pH 7–8) are

also consistent with this interpretation (Fig. 2). The values of k_2 obtained by Jones *et al.* (1973) between pH8.5 and pH9.5 were lower than those expected from an inverse linear dependence on $[H^+]$. These results were, however, obtained in carbonate buffers and the discrepancy may be due to specific buffer effects. The inverse dependence of k_2 on $[H^+]$ up to at least pH 10 suggests the involvement of a process with $pK \geqslant 10.5$. This is too high for any known reaction involving the ferrihaem and it is likely to be due to the dissociation of H_2O_2 ($pK = 11.6$; Kelly *et al.*, 1977). This pH-dependence may be interpreted in terms of a pre-equilibrium dissociation of H_2O_2 followed by attack by HO_2^- on ferrihaem monomer (Scheme 2). Eqn. (4) may then be rewritten as:

$$R_i = k_2^0[M]\,[HO_2^-] \qquad (5)$$

where k_2^0 is a pH-independent second-order rate constant. Values of k_2^0 (eqn. 5) calculated from the intercepts of plots of log k_2 against pH are shown in Table 1. The data for deuteroferrihaem at I 0.05 and I 0.1 show that k_2^0 decreases with increasing ionic strength, probably owing to a specific inhibitory effect of phosphate buffer anions. Such an effect might also be expected for protoferrihaem, which would suggest that the value of k_2^0 for protoferrihaem at I0.05 would be somewhat higher than the values for deuteroferrihaem and mesoferrihaem.

Comparison of the rate-dependence and kinetic parameters obtained in the present study with those observed in the study of the decomposition of H_2O_2 (Hatzikonstantinou & Brown, 1978) reveals striking similarities. In both systems the rate is carried mainly by the monomeric species and the pH-dependences of the monomer activities are identical. The specific pH-independent rate constants follow the same sequence in both systems, i.e. coproferrihaem > protoferrihaem > mesoferrihaem ≈ deuteroferrihaem. These findings strongly suggest that the initial reactions in both systems are identical. Kelly *et al.* (1977) have obtained data for deuteroferrihaem consistent with the peroxidatic theory of catalase action (Chance *et al.*, 1952) involving the initial formation of a complex between ferrihaem and HO_2^- followed by irreversible formation of a peroxidatic intermediate equivalent to catalase–H_2O_2 Compound I (Scheme 2). It seems possible that the initial complex, M*–OOH^-, may also be involved



Scheme 2. *Various reactions involving ferrihaems, H_2O_2 and O_2*
The scheme envisages a competition for the initial complex M*–OOH^-, which may be converted either into the peroxidatic intermediate or to bile pigment, via the secondary complex M–OOH^-. This secondary complex may also be formed during haem catabolism by the action of reducing agent on a haem–O_2 complex.

Table 1. *pH-independent rate constants for the oxidation of ferrihaems by H_2O_2*
The values of a_M^0, the pH-independent second-order rate constant for decomposition of H_2O_2, were taken from Hatzikonstantinou & Brown (1978). The values of m were determined from the slopes of graphs such as that shown in Fig. 7.

Ferrihaem	Ionic strength (M)	$10^{-7} \times k_2^0$ ($M^{-1} \cdot min^{-1}$)	$10^{-7} \times a_M^0$ ($M^{-1} \cdot min^{-1}$)	$\frac{2a_M^0}{k_2^0}$	m
Coproferrihaem	0.05	13.3	100	16	19
Mesoferrihaem	0.05	2.5	28	23	27
Deuteroferrihaem	0.05	2.9	28	20	29
Deuteroferrihaem	0.10	0.9	—	—	—
Protoferrihaem	0.10	2.5	64	48	45

in a reaction leading to ferrihaem degradation as shown in Scheme 2. Such a scheme would automatically explain the similarities in the decomposition and ferrihaem-degradation reactions. Kelly *et al.* (1977) suggested that the observed catalytic effect of phosphate buffer anions on decomposition might be due to a general acid catalysis on the step in Scheme 2 where the peroxidatic intermediate is produced. Since there is a competition for M*–OOH^-, such a catalysis would result in a consequent inhibition in the rate of production of bile pigment as observed in the present work.

The magnitude of the k_2^0 values in Table 1 are smaller than the corresponding rate constants for H_2O_2 decomposition (Hatzikonstantinou & Brown, 1978) by factors of between 8 and 25. Taking coproferrihaem as an example, this implies that for every eight molecules involved in the catalytic cycle, one molecule proceeds along the degradation pathway. On average, therefore, each ferrihaem molecule can complete about eight turns of the cycle before being degraded, corresponding to decomposition of sixteen H_2O_2 molecules. In general:

$$\Delta[H_2O_2] = m[T] \quad (6)$$

where $\Delta[H_2O_2]$ represents the maximum concentration of H_2O_2 decomposed and m is a constant for any given ferrihaem. By using the above arguments, m is related to the relevant rate constants by

$$m = 2a_M^0/k_2^0 \quad (7)$$

where a_M^0 is the pH-independent second-order rate constant for decomposition of H_2O_2. Since all of the decomposition-rate measurements used in the present work were continued until catalytic decomposition had ceased, this corollary to Scheme 2 may be tested against the experimental data. Fig. 6 shows the values of $\Delta[H_2O_2]$ as a function of initial ferrihaem concentration for coproferrihaem. The data are in reasonable agreement with a linear relationship that is almost independent of pH as required. The slope of the line (m) in Fig. 6 is 19, compared with the predicted value from the rate constants of 16. For deuteroferrihaem, mesoferrihaem and protoferrihaem, similar relationships were found and the appropriate values of m are shown in Table 1. The agreement between these values (which do not require rate measurements) and the predictions from the values of the rate constants (eqn. 7) is reasonably good. This finding lends support to the postulates embodied in Scheme 2 and, in addition, shows that the data are internally consistent.

Although no proof is presently available, it has been suggested (Brown, 1976; Brown & Grundy, 1977; King & Brown, 1978) that haem catabolism involves intramolecular attack by activated molecular oxygen, probably at the peroxide level. In this event,

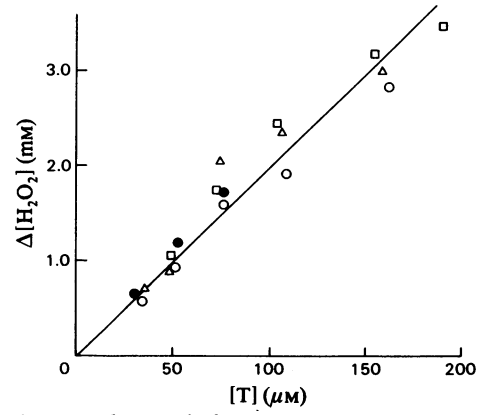


Fig. 6. *Dependence of the maximum concentration of H_2O_2 decomposed on coproferrihaem concentration*
$\Delta[H_2O_2]$ represents the extent of decomposition when [T] has been decreased to zero. □, pH7.74; ●, pH7.56; △, pH7.35; ○, pH6.81. The slope of this line (m) is 19 (see the text and Table 1).

it is possible that haem degradation by H_2O_2 may also be an intramolecular reaction and that the complex containing activated oxygen may be the same whether the primary agent of degradation is H_2O_2 or molecular oxygen with reducing agent (haem catabolism). However, there is no evidence for the formation of a peroxidatic intermediate during haem degradation by molecular oxygen and a reducing agent. For this reason, an irreversible step has been postulated in Scheme 2 (M*–$OOH^- \rightarrow$ M–OOH^-) which attempts to correlate the various reactions between haem, molecular oxygen and H_2O_2, relevant to the present work. It seems likely that hydroxylation reactions catalysed by cytochrome *P*-450 may also involve a similar activated oxygen complex to that shown in Scheme 2.

The findings of the present work suggest that ferrihaem oxidation by H_2O_2 may represent an accurate model for haem catabolism. It would be useful therefore to examine the mechanism of this reaction further with methods used in the study of haem catabolism, e.g. ^{18}O studies (Brown & King, 1978). Unfortunately, a stoicheiometric excess of H_2O_2 is required because of catalytic decomposition and this leads to further oxidation of the product bile pigment. Such a situation does not arise in the systems using molecular oxygen and reducing agent, since the oxygen appears to be reduced in intramolecular association with haem and no free H_2O_2 is released.

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