Kinetics and Mechanism of a Chemiluminescent Clock Reaction based on the Horseradish Peroxidase Catalysed Oxidation of Luminol by Hydrogen Peroxide

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In the presence of limited amounts of urate the rapid onset of light emission from the horseradish peroxide catalysed oxidation of luminol by hydrogen peroxide is delayed, yielding a chemiluminescent clock reaction. Under appropriate conditions the rate of urate oxidation by hydrogen peroxide in the dark reaction is independent of the concentration of both reagents, reflecting a steady state in the catalytic system in which the enzyme intermediate, Compound (II), preferentially reacts with luminol to yield luminol radicals which rapidly oxidise urate. Luminol, therefore, acts as an electron-transfer mediator during the dark reaction and its concentration remains constant, yielding an overall zero-order process.

The classical 'Iodine Clock' and diverse similar clock reactions have intrigued countless young (and not-so-young) students of chemistry for many years. The principle of *in situ* titration employed in these reactions, in which the titrant is added *via* its generation as the product of a chemical reaction (*e.g.* iodine in the 'Iodine Clock' reactions) also provides an analytical technique which can form the basis of kinetic studies.

Chemiluminescent reactions, for example, the chemiluminogenic oxidation of luminol, are also a long-standing feature of the literature on entertaining demonstration experiments. More recently chemiluminscent methods of analysis have acquired increasing importance[1] as a consequence of their high intrinsic sensitivity (using photomultiplier detection) and the desirability of replacing methodologies dependent on the use of radioactive materials.

Combination of the 'clock reaction' principle with a chemiluminogenic reaction led to the development of a 'chemiluminescent clock reaction' demonstration experiment in which the end point is marked by a flash of light from the solution.[2] This reaction employs the oxidation of luminol by hydrogen peroxide, catalysed by the iron(III) porphyrin complex deuteroferrihaem,[3] in which the preferential oxidation in the dark reaction of limited amounts of urate or ascorbate delays the onset of the chemiluminescent end-point reaction. Using photomultiplier detection this technique has analytical applications in which the time delay to the onset of chemiluminscence is a parameter that is dependent only on the chemistry of the sample.[4] In contrast, in conventional chemiluminometric assays, the emitted light intensity (measured either as peak intensity or the integral over an arbitrary time period) is the analytical parameter and the results obtained depend on the design, geometry and stability of the apparatus and on the time course of the light emission (including problems associated with mixing artifacts).

Deuteroferrihaem is a more effective catalyst of the luminol oxidation reaction than protoferrihaem (haemin), largely because it shows a much lower tendency to dimerise in aqueous solution.[5] However, the kinetics of the catalysis by both haem complexes are complicated and markedly pH dependent.[3,6] In the present work the haem enzyme horseradish peroxidase has been used as the catalyst for luminol oxidation and kinetic studies are reported for experiments in which urate has been employed as the delay reagent. The general mechanism of peroxidase catalysis is well understood[7] and specific aspects of catalysis in luminol oxidation[8] and post-catalytic processes in the light emitting reaction have also been discussed.[9]

Experimental

Materials.—Triply distilled water was used in the preparation of solutions. Horseradish peroxidase (Type VI) was obtained from Sigma. The concentration of a stock solution (7.5 mg of enzyme in 10 cm^3 water) was monitored spectrophotometrically [ε(403 nm) 1.02 × 10^5 dm^3 mol^{-1} cm^{-1}].[10] Hydrogen peroxide was supplied as 35% unstabilised solution by Interox and 0.1 mol dm^{-3} stock solutions were assayed cerimetrically. More dilute reaction solutions were prepared daily by quantitative dilution. Luminol (Sigma) was used without further purification; stock solutions (*ca.* 18 mmol dm^{-3}) were prepared by dissolving the required amount of luminol in a small volume of 1 mol dm^{-3} sodium hydroxide solution and making up with water. More dilute reaction solutions were prepared daily by quantitative dilution. Solutions of uric acid (Sigma) were prepared immediately prior to use in water saturated with O_2-free nitrogen. All experiments were carried out at pH 7, phosphate buffer, ionic strength 0.1 mol dm^{-3} (adjusted with $NaNO_3$).

Methods.—Chemiluminescence measurements employed an LKB 1250 luminometer with the base of the cuvette holder modified to accommodate a microstirrer. Reaction solutions were incubated at 25 °C before use. Small volumes of peroxidase, luminol, and urate solutions were injected from micropipettes on to the base and opposite walls of the cylindrical, round-bottomed luminometer tubes, stirring was initiated and the reaction started by the rapid injection of 2 cm^3 of buffered hydrogen peroxide solution. The output signal from the luminometer was taken to a Servoscribe Y-t recorder and the time delay (T) from reaction initiation to the sharp onset of chemiluminescence was recorded.

Stopped-flow measurements (Durrum D110) of the reactions of pre-formed enzyme intermediates [the Compound (I) and Compound (II) intermediates of horseradish peroxidase[7]] with luminol and urate at pH 7, 25 °C were made under pseudo-first-order conditions, following the procedures described by Dunford and co-workers.[11,12]

Results and Discussion

Systematic studies of the variation of T with changes in initial concentrations of horseradish peroxidase, [E_t], hydrogen peroxide, [P], urate, [U], and luminol, [L], were made. The results may be summarised as follows. (*i*) At constant [E_t], [P], and [L], T is proportional to [U] [Figure 1(*a*)]. This implies that the rate of oxidation of urate during the dark reaction is

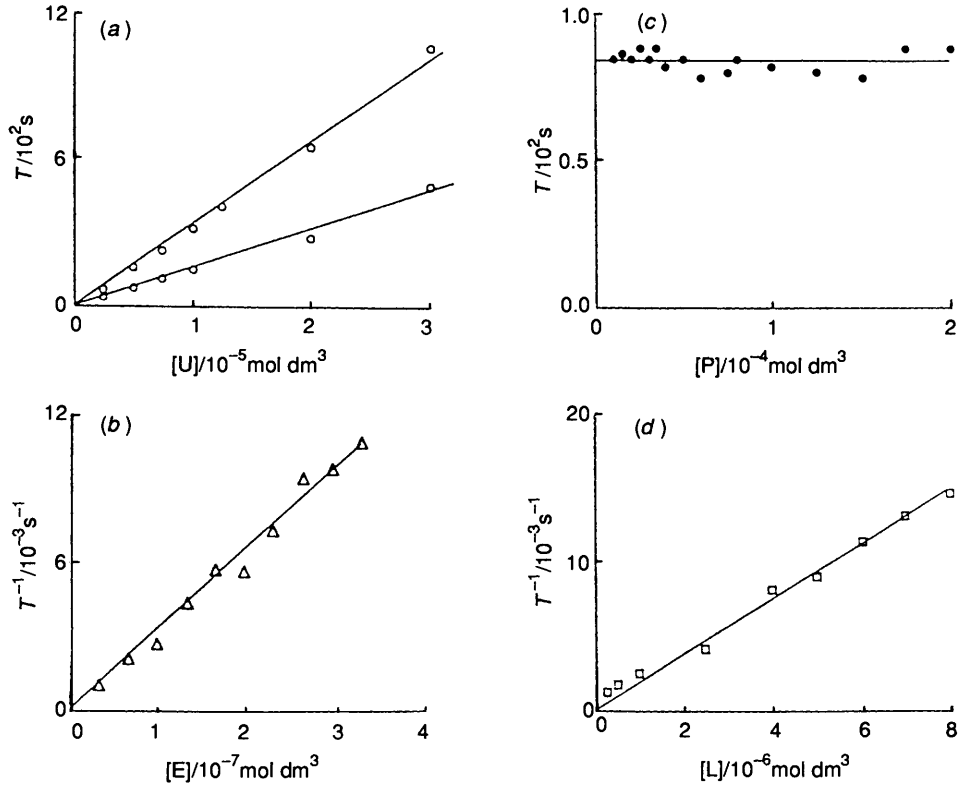

Figure 1. Dependence of the clock reaction delay (T) on initial concentrations of horseradish peroxidase, [E_t], hydrogen peroxide, [P], luminol, [L], and urate, [U]. (*a*) Dependence on [U] using [P] = 40 μmol dm^{-3}, [L] = 4.95 μmol dm^{-3}, and [E_t] = 0.133 μmol dm^{-3} (lower curve), [E_t] = 0.265 μmol dm^{-3} (upper curve). (*b*) Dependence on [E_t] using [P] = 40 μmol dm^{-3}, [L] = 4.95 μmol dm^{-3}, [U] = 7.5 μmol dm^{-3}. (*c*) Dependence on [P] using [E_t] = 0.265 μmol dm^{-3}, [U] = 5.5 μmol dm^{-3}, [L] = 4.95 μmol dm^{-3}. (*d*) Dependence on [L] using [E_t] = 0.265 μmol dm^{-3}, [P] = 40 μmol dm^{-3}, [U] = 7.5 μmol dm^{-3}.

independent of [U]. (*ii*) At constant [U], [P], and [L], $1/T$ is directly proportional to [E_t] [Figure 1(*b*)]. (*iii*) At constant [E_t], [U], and [L], T is independent of [P] [Figure 1(*c*)]. (*iv*) At constant [E_t], [U], and [P], $1/T$ is directly proportional to [L] over most of the range studied [Figure 1(*d*)] although at lower [L] the values of $1/T$ lie somewhat higher than the straight line through the origin.

Results (*i*) and (*iii*) together imply that the rate of oxidation of urate by hydrogen peroxide during the dark reaction is independent of the concentration of both species. Measurements of the peak light intensity of the end-point reaction, I, showed that I varies linearly with [P], at constant [E_t], [U], and [L], yielding an intercept on the abscissa which is consistent with a 1:1 stoicheiometry in the dark reaction oxidation of urate by hydrogen peroxide [Figure 2(*a*)]. At constant [U], [P], and [L], I is proportional to [E_t] [Figure 2(*b*)]. The data in Figure 2(*c*) shows the variation of I with [U] at constant [E_t], [P], and [L]. Assuming a 1:1 reaction stoicheiometry and proportionality between light intensity and hydrogen peroxide concentration [Figure 2(*a*)], the variation of I with [U] is given by $I = I_0(1 - [U]/[P])$ where I_0 is the intensity when [U] = 0.

The general mechanism of horseradish peroxidase catalysis has been applied by Cormier and Prichard [8] to the oxidation of luminol, equations (1)–(3), where (**I**) and (**II**) are the oxidising

$$E + P \xrightarrow{k_1} (I) \quad (1)$$

$$(I) + L \xrightarrow{k_2} (II) + L^{\bullet} \quad (2)$$

$$(II) + L \xrightarrow{k_3} E + L^{\bullet} \quad (3)$$

enzyme intermediates Compounds (**I**) and (**II**) respectively [7] and L$^{\bullet}$ is the luminol radical formed by one-electron oxidation of L. The subsequent fate of L$^{\bullet}$ in the chemiluminescent reaction has been elaborated by Merenyi and Lind.[9] For the present purpose this post-catalytic process can be represented by equation (4), in which disproportionation of L$^{\bullet}$ yields L and

$$L^{\bullet} + L^{\bullet} \xrightarrow{k_4} L + A \quad (4)$$

$$A + HO_2^- \longrightarrow [\text{Aminophthalate}]^* \longrightarrow \text{Light}$$

the luminol azaquinone (A). The latter species undergoes reaction with the anion of P (HO_2^-), resulting in a series of processes which yields the excited state aminophthalate emitter.

In the presence of urate as a donor substrate reaction of (**I**) and (**II**) with U can occur, equations (5) and (6), where U$^{\bullet}$ is the

$$(I) + U \xrightarrow{k_5} (II) + U^{\bullet} \quad (5)$$

$$(II) + U \xrightarrow{k_6} E + U^{\bullet} \quad (6)$$

urate radical formed by one-electron oxidation of U. Overall urate undergoes a two-electron oxidation by hydrogen peroxide, possibly *via* disproportionation of U$^{\bullet}$.

Although this model implies that, in the clock reaction, L and U are in competition for (**I**) and (**II**) and that light emission may, therefore, be attenuated in the presence of U, such a competition process does not readily account for the complete abolition of light emission followed by a very rapid onset of chemiluminescence and indeed the rate constant data (Table)

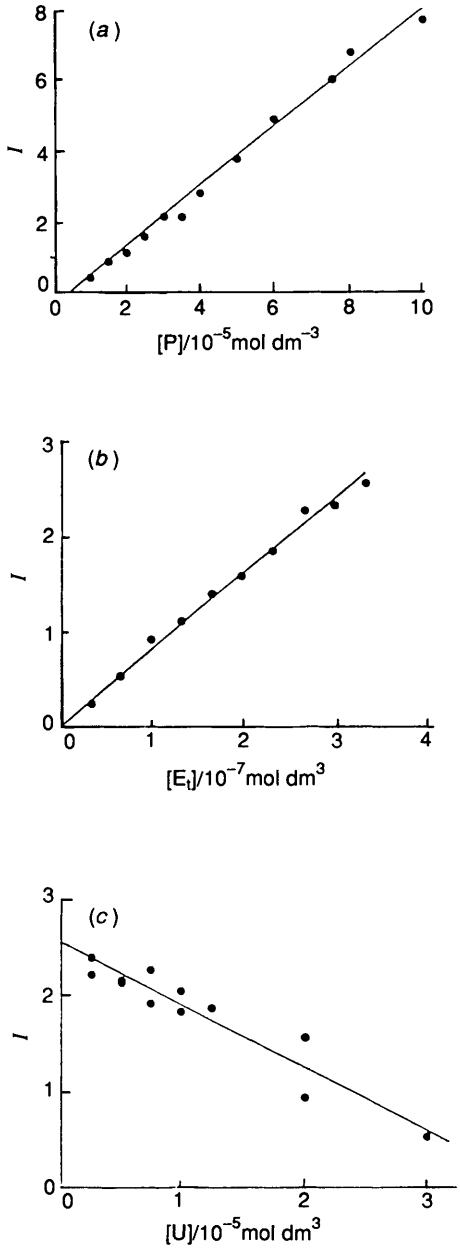

Figure 2. Dependence of post-delay chemiluminescence intensity, I, on: (*a*) [P], conditions as in Figure 1(*c*); (*b*) [E_t], conditions as in Figure 1(*b*); (*c*) [U], conditions as in Figure 1(*a*) (values of I have been normalised, to take into account the difference in [E_t] using the results of Figure 1(*b*); the slope of the solid line corresponds to 1:1 stoicheiometry in the oxidation of urate by hydrogen peroxide. Values of I are measured relative to an instrument internal standard.)

imply that it is not a significant effect under the conditions employed. Furthermore, this model does not lead to an inverse dependence of T on [L]. The latter result can be accommodated by proposing that U reduces the luminol radical according to equation (7). Steady-state analysis of the mechanism represented

$$L^{\cdot} + U \xrightarrow{k_7} L + U^{\cdot} \qquad (7)$$

by equations (1)–(7), taking into account the conservation of enzyme according to equation (8) leads to equation (9) for the

$$[E_t] = [E] + [(I)] + [(II)] \qquad (8)$$

Table. Rate constants determined at 25 °C, pH 7 by stopped-flow spectrophotometry.

Reaction	Rate constant/dm^3 mol^{-1} s^{-1}
(1)	$k_1 = 2 \times 10^7$ [a]
(2)	$k_2 = 6.5 \pm 0.2 \times 10^5$
(3)	$k_3 = 2.3 \pm 0.3 \times 10^4$
(5)	$k_5 = 1.3 \pm 0.1 \times 10^4$
(6)	$k_6 = 8.2 \pm 0.1 \times 10^2$

[a] Reference 13, other values, this work.

$$\frac{-d[U]}{dt} = \frac{k_1[P][E_t](k_5[U] + k_6 f[U] + k_2[L] + k_3 f[L])}{(k_2[L] + k_5[U]) + \{k_1[P](1 + f)\}} \qquad (9)$$

$$f = (k_2[L] + k_5[U])/(k_3[L] + k_6[U]) \qquad (10)$$

rate of oxidation of urate. Rate constants for reactions (1), (2), (3), (5), and (6), under the conditions employed in the steady-state experiments, have been obtained from stopped-flow studies and the results are shown in the Table. In studies of the reactions of (**I**) and (**II**) with L the reactions occurring are (2) + (4) and (3) + (4) respectively, equations (11) and (12). Similarly

$$-d[(I)]/dt = 2k_2[(I)][L] \qquad (11)$$

$$-d[(II)]/dt = 2k_3[(II)][L] \qquad (12)$$

in the studies of the reduction of (**I**) and (**II**) by U the observed rate constants are $2k_5$ and $2k_6$ respectively. The rate constant data may be used to simplify equation (9) under the experimental conditions employed in the clock reactions. The second term in the denominator of (9) is dominant so that $-d[U]/dt$ becomes independent of [P]. This is a usual situation in peroxidase catalysis where the relatively high rate constants for reaction (1) and for reactions of (**I**) *vis-à-vis* reactions of (**II**) with donors result in a compound (**II**) limited overall reaction in the steady state. Since $k_2 \gg k_3$ and $k_5 \gg k_6$, equation (9) then simplifies to equation (13). In almost all the clock experiments,

$$-d[U]/dt = 2[E_t](k_3[L] + k_6[U]) \qquad (13)$$

except for a few at very low [L] [Figure 1(*d*)], it is also the case that $k_3[L] \gg k_6[U]$ so that equation (13) further simplifies to equation (14). Equation (14) implies that the rate of urate

$$-d[U]/dt = 2k_3[E_t][L] \qquad (14)$$

oxidation during the dark reaction is independent of [U] and [P] (as observed). The value of [L] remains constant during the dark reaction (it is merely acting as an electron-transfer mediator), as does [E_t] so that overall the reaction is zero order.

Therefore, the delay time may be expressed as equation (15). The validity of equation (15) may be tested quantitatively using

$$T = (1/2k_3)[U]/[E_t][L] \qquad (15)$$

the steady-state data (Figure 1) and the independently determined value of k_3 (Table) as shown in Figure 3. The results show that the delay time in the dark reaction is predictable on the basis of equation (15) except at very low [L] relative to [U] where the values of T are somewhat smaller than predicted [Figure 1(*d*)] since direct oxidation of U by (**II**) [equation (6)] now becomes significant [equation (13)].

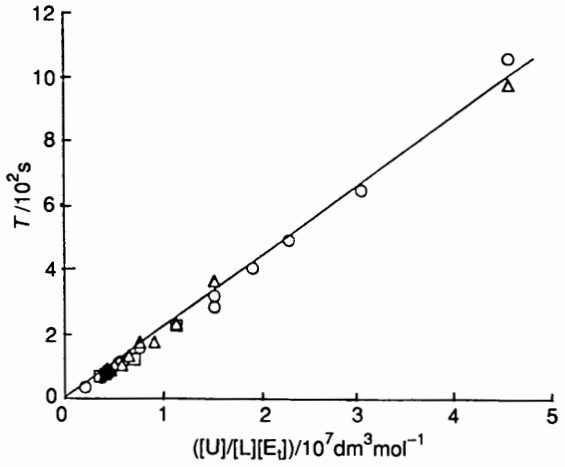

Figure 3. Data of Figure 1 plotted according to equation (15). The solid line has a slope of $1/2k_3$ (Table). Results obtained at the lowest values of [L] in Figure 1(*d*) have been omitted (see the text).

In clock reactions *in situ* titration is usually employed in the study of reaction kinetics, as in the present work. Since equation (15) affords a quantitative description of the concentration dependence of T, the possibility arises of using the system in the reverse sense, *i.e.* determining reactant concentration from measurements of T. Of particular interest would be measurements of low peroxidase concentrations. Values of $[U]/[L] = 10^{-3}$ are practicable, with $[U] = 10^{-6}$ mol dm^{-3} and, under such conditions, equation (15) predicts that 3.6×10^{-10} mol dm^{-3} peroxidase would yield T 1 min. Tests of the practicality of such measurements are in progress.

Acknowledgements

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