On the Mechanism of Peroxidase Catalyzed Oxidations Studied by Means of Chemiluminescence Measurements

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The enzymic oxidation of pyrogallol is accompanied by chemi-luminescence, the measurement of which provides a sensitive and useful assay method for peroxidatic activity of purified as well as for crude enzyme preparations.

The present investigation is concerned with the characteri-zation and kinetics of the luminescent species involved in this reaction.

It has long been known that the oxidation of pyrogallol is accompanied by chemiluminescence in the presence of hydrogen peroxide [1,2]. However, no attempts have been made to study the reaction mechanism of this process in further detail. In a previous publication the possibilities of utilizing this light emission for the quantitative measurement of peroxidatic activity were investigated. It was found that when pyrogallol, phosphate buffer and enzyme were mixed, the light intensity reached a maximum value after a certain induction period, and then slowly decreased.[3] The form of the time dependence reaction velocity curves for the luminescent process strongly suggested the accumulation of an active intermediate molecular species which then undergoes a secondary, chemiluminescent elimination without necessarily requiring the participation of the enzyme protein. The luminescence intensity at the "steady state" was found to be dependent on the enzyme concentration, but the relationship was linear only within a very limited concentration range of oxidizing substrate. The luminescence at the maximal "steady state" conditions was further characterized by a marked sensitivity to small variations in peroxide concentration and possessed a sharp optimum. The position of this optimum was found independent of enzyme concentration. The presence of a large excess of reducing substrate resulted in an inhibition of the luminescent reaction which has previously been observed for the over-all reaction rate.[4] The advantages of this method as compared to the conventional assay methods were also presented. In the present experimental work, an attempt has been made to clarify the reaction mechanism of the luminescent process in further detail.

EXPERIMENTAL

The equipment used for the luminescence measurements consisted of a sensitive photomultiplier unit coupled to a potentiometer recorder. A Sigma HRP (horse-radish peroxidase) preparation of a purity corresponding to 21 purpurogallin units[5] per mg (20 sec) was used. All other chemicals were analytically pure. In order to prevent autoxidation, the freshly prepared pyrogallol solutions were immediately acidified by the addition of a few drops of 1 M sulphuric acid.

Pyrogallol, phosphate buffer and enzyme were mixed in a standard cuvette in the above mentioned order, and the volume of the reaction mixture adjusted to 1.5 ml by the addition of distilled water. The reaction was then initiated by the rapid injection of 1.0 ml of peroxide into the reaction chamber which ensured an immediate and efficient mixing of the components. The luminescence intensity was then recorded as a function of time under varying experimental conditions.

The light absorption measurements were carried out under identical experimental conditions by means of a Beckman spectrophotometer unit type DB.

RESULTS AND DISCUSSION

The effect of pH and temperature on the luminescence intensity at the steady state

The dependence of the luminescence intensity on hydrogen ion concentration at the maximal steady state conditions in the pH interval 4.0—9.0 is given in Fig. 1. A fairly sharp optimum is observed between pH 6.0 and 6.5, whereas the pH optimum as obtained by the purpurogallin method is reported to lie around pH 7.8.[6] By the use of monochromatic interference filters, the light emitted was shown to have a maximum intensity at a wavelength near 4800 Å.
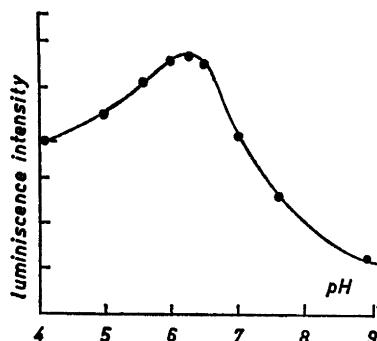

Fig. 1. The dependence of the luminescence intensity on pH under maximum "steady state" conditions. HRP $= 3.6 \times 10^{-8}$ M. Pyrogallol $= 6.3 \times 10^{-4}$ M. $H_2O_2 = 3.5 \times 10^{-2}$ M.

From measurements of the temperature dependence of the luminescent process in the temperature range 10 to 20°C, an apparent activation energy of approximately 8 400 cal mole^{-1} was obtained. This value is considerably lower than that which may be calculated from the figures reported by Willstätter and Weber[7] using leuco-malachite green as hydrogen donor. In the latter case an activation energy of about 11 500 cal mole^{-1} will be found for the over-all activity of the enzyme in the same temperature range. The

formation of malachite green from the leuco-base closely agrees with the reaction kinetics of the rate determining enzyme-substrate complexes, and thus constitutes a more reliable index of the true enzymic activity, than is given by the purpurogallin number.[8] The luminescence is clearly associated with a secondary, non-enzymic reaction step in the oxidation of pyrogallol of much lower energy of activation than the enzymic reactions.

The spectrophotometric identification of an intermediate product formed by the enzymic oxidation of pyrogallol

In order to confirm our hypothesis concerning the existence of an active, chemiluminescent intermediate which is eliminated by a process slower than the enzymic reaction, the formation of end products in time was studied spectrophotometrically at different wavelengths. When the light absorbtion of the reaction mixture was registered at 6000 Å, it was found, that the optical density, instead of displaying the continuous increase reflecting the accumulation of coloured end products, reached a maximal level after an induction period of several minutes and then decreased, asymptotically approaching a much lower, constant value. Thus, it could be established, that during the course of the reaction, a blue, intermediary compound accumulates which then is eliminated by a slower, secondary reaction.

To investigate whether the blue compound in some way was associated with the luminescent process, the light intensity was recorded under identical experimental conditions. It was found, that the position of the maximal light intensity at pH 7.0 and 6.2 coincides with the position of the maximal light absorption. However, after the maximum had been reached, the optical density decreased somewhat slower than that observed for the light emission, and when the luminescence had practically ceased, the absorbancy was still considerable, amounting to approximately one third of the maximum. We attributed this discrepancy between the changes in absorbancy and light intensity, which becomes increasingly more accentuated as the reaction approaches its end point, to the formation of end products also causing some light absorption at this wavelength. This assumption could be proved by the following observations.

By the addition of a strong reducing agent, e.g. ascorbic acid, an immediate decrease of the absorption at 6000 Å was obtained, whereas the absorbancy at 4300 Å, the wavelength usually employed[9] for the registration of end products, was not appreciably altered. Under the same conditions ascorbic acid was also found to cause an immediate and complete extinction of the chemiluminescence which is shown in Fig. 2. The absorbancy values, obtained after the reducing agent has been added at different time intervals, thus represent the relative amounts of end products irreversibly formed. Under the experimental conditions in question, the formation velocity of these end products (presumable mostly purpurogallin) is constant during the first period of time, and then gradually decreases as the supply of initial reactants is consumed. This characteristic will also apply to the formation of purpurogallin as usually registered in the spectrophotometric adaptions of the conventional purpurogallin method
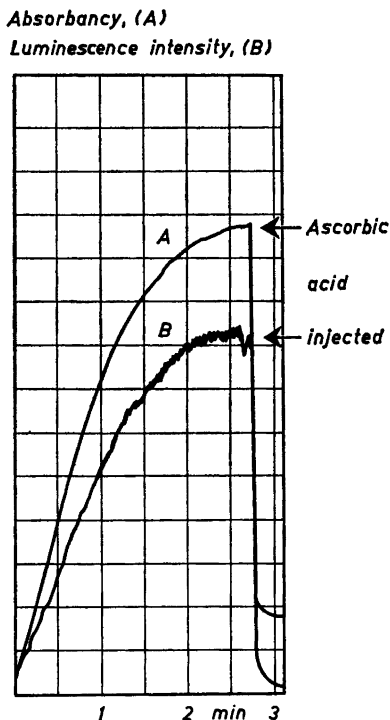
Absorbancy, (A)
Luminescence intensity, (B)

Absorbancy (A)
Luminescence intensity (B)

Fig. 2. The effect of ascorbic acid on the concentration of the blue compound and on the luminescence intensity. HRP = 3.6×10^{-8} M. Pyrogallol = 6.3×10^{-4} M. $H_2O_2 = 7.0 \times 10^{-3}$ M. pH = 7.0.
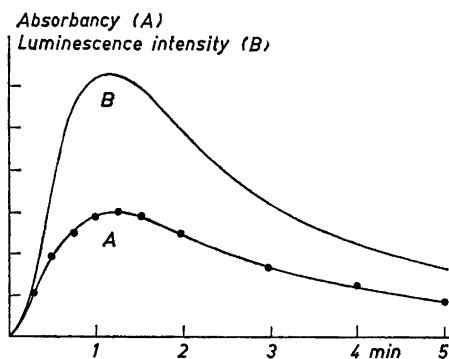
Fig. 3. The variations in time of the absorbance caused solely by the blue intermediate, plotted together with the variations in luminescence intensity measured under identical conditions. HRP = 3.6×10^{-8} M. Pyrogallol = 6.3×10^{-4} M. $H_2O_2 = 3.5 \times 10^{-2}$ M. pH = 7.0.

of Willstätter. The differences in absorbancy at 6000 Å, obtained by subtracting the values observed after the injection of the ascorbic acid from the values observed before the reducing agent was added, thus represent the absorption solely caused by the blue compound. When the variation in absorbancy induced by this readily reducible intermediate is plotted against time in a diagram together with the variations in luminescence intensity, a good correlation between the two curves is obtained, as shown in Fig. 3.

The effect of different variables on the formation and elimination of the blue intermediate
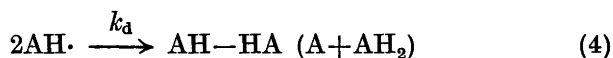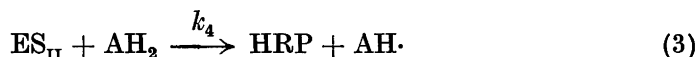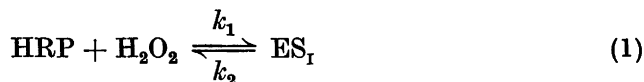
Effect of pH. To determine the position of the luminescent step and its relation to the formation and elimination of the blue compound in the reaction sequence resulting in the formation of purpurogallin, the influence of pH

on the correlation of the luminescence and the levels of the intermediate was studied. Whereas at pH 6.2 and 7.0 there is a close correspondence in time between these two curves, the luminescence peak at pH 7.6, however, considerably lags behind the position of the maximum in the concentration of the blue intermediate. Another interesting feature is that the hydrogen ion concentration was found to have a relatively small effect on the levels of the blue compound in the pH region where the luminescence had a marked optimum. Consequently, neither the formation of the blue compound, nor its elimination, is identical with the luminescent reaction. This might be understood more clearly on basis of the following considerations.

If the formation of the blue compound is identical with the luminescent reaction then the maximum of the light emission (indicating a maximum turnover rate of the immediate precursor of the blue compound) would precede the position of the maximum of the concentration of the intermediate, when the rate of formation of the latter equals the rate of its elimination. If, on the other hand, the luminescent reaction is identical with the elimination of the intermediate, the maximum in luminescence should correspond to a maximal concentration level of this compound. Thus, both the formation and the elimination of the intermediate precedes the pH dependent luminescent step.

The effect of peroxide and pyrogallol concentrations. In order to gain more concrete information on the true nature of the blue intermediate, an investigation was undertaken of the effect of certain variables known to affect the levels of the intermediate such as enzyme and peroxide concentrations.

By means of electron paramagnetic resonance measurements, Yamazaki, Mason and Piette have identified free radicals formed from various hydrogen donors by one-electron-reductions of the active primary and secondary peroxidase-peroxide complexes. The general reaction mechanism proposed for the oxidation of a hydrogen donor, AH_2, may be written as [10-12]

$$HRP + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_I \tag{1}$$

$$ES_I + AH_2 \overset{k_7}{\longrightarrow} ES_{II} + AH\cdot \tag{2}$$

$$ES_{II} + AH_2 \overset{k_4}{\longrightarrow} HRP + AH\cdot \tag{3}$$

$$2AH\cdot \overset{k_d}{\longrightarrow} AH-HA \ (A+AH_2) \tag{4}$$

where $AH\cdot$ represents the (semi)—oxidized donor molecule formed by the reduction of the active complexes ES_I and ES_{II}. The reaction between complex II and the donor molecules is usually the rate determining step. From these equations we obtain the following rate expressions:

$$d[ES_I]/dt = [E] [H_2O_2] k_1 - [ES_I] (k_2 + [AH_2] k_7) \tag{5}$$

$$d[ES_{II}]/dt = [ES_I] [AH_2] k_7 - [ES_{II}] [AH_2] k_4 \tag{6}$$

$$d[AH\cdot]/dt = [ES_I] [AH_2] k_7 + [ES_{II}] [AH_2] k_4 - [AH\cdot]^2 k_d \tag{7}$$

If the steady state conditions $d[ES_I]/dt = d[ES_{II}]/dt = 0$ are applied, we obtain from eqns. (5) and (6):

$$[ES_I] = \frac{[E_0]\ [H_2O_2]\ k_1 - [ES_{II}]\ [H_2O_2]\ k_1}{k_2 + [AH_2]\ k_7 + [H_2O_2]\ k_1} \tag{8}$$

$$[ES_{II}] = \frac{[E_0]\ [H_2O_2]}{[H_2O_2]\ (1 + k_4/k_7) + k_4/k_1(k_2/k_7 + [AH_2])} \tag{9}$$

where $[E_0]$ represents the total enzyme concentration. As k_2/k_7 is much smaller than $[AH_2]$, and k_7, as far as investigation of this constant is possible, has been shown to be much greater than k_4 for HRP, eqn. (9) may be simplified to

$$[ES_{II}] = \frac{[E_0]}{1 + [AH_2]\ k_4/[H_2O_2]\ k_1} \tag{10}$$

If it is assumed, that the blue intermediate is identical with the free radical directly formed by the reduction of the active enzyme-substrate complexes, the expression for the steady state concentration of this compound should agree closely with that of complex II and will be given by expression (11):

$$[AH\cdot] = \left[\frac{2[E_0]}{\dfrac{k_d}{k_1[H_2O_2]} + \dfrac{k_d}{k_4[AH_2]}} \right]^{\frac{1}{2}} \tag{11}$$

Eqn. (11) will be valid under steady state conditions, *i.e.* $d[AH\cdot]/dt = 0$, provided that the free radicals disappear only by reaction with one another. However, in the case of pyrogallol, the course of reaction is considerably more complicated, and one or more steps, involved in the formation of end products, are slower than the reaction between the hydrogen donor and complex II.[3,13] Thus several alternative mechanisms of elimination of the primarily formed free radicals are possible.

The dependence of the initial formation velocity of the intermediate on the enzyme concentration at different levels of oxidizing substrate was
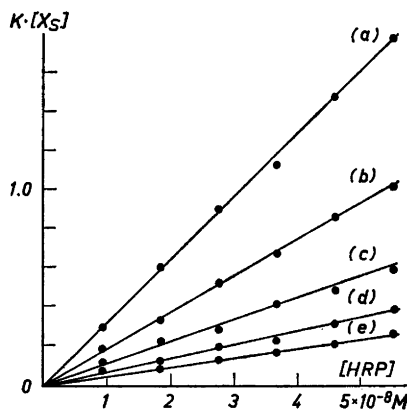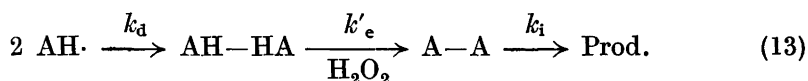

Fig. 4. The dependence of the concentration of the blue compound on the enzyme concentration at five different levels of oxidizing substrate. Pyrogallol $= 6.3 \times 10^{-4}$ M. $H_2O_2 = $ (a) 7.0×10^{-3}, (b) 10.5×10^{-3}, (c) 17.5×10^{-3}, (d) 26.2×10^{-3}, and (e) 35.0×10^{-3} M. pH $= 7.0$.

investigated. The relationship was found to be linear, which is consistent with the blue compound being identical with the free radical. This evidence can, however, as well be interpreted on the basis of the intermediate being identical with a dimerization product of the radical. In order to decide between the two alternatives, the influence of the enzyme concentration on the steady state levels of the blue compound at different peroxide concentrations was studied. The results of this investigation are represented in Fig. 4. For none of the five curves is the steady state concentration of the intermediate proportional to $[E_0]^{\frac{1}{2}}$, and the intermediate cannot, thus, be identical with the free radical. The steady state concentrations are directly proportional to the enzyme concentration which satisfies an expression representing the steady state concentration of a hypothetical recombination product formed from the free radicals, either with or without the participation of peroxide, as shown below in the reaction sequences (12) and (13).

$$2 \text{ AH} \cdot \xrightarrow{k_d} \text{AH}-\text{HA} \xrightarrow{k_e} \text{Prod.} \tag{12}$$

$$2 \text{ AH} \cdot \xrightarrow{k_d} \text{AH}-\text{HA} \xrightarrow[\text{H}_2\text{O}_2]{k'_e} \text{A}-\text{A} \xrightarrow{k_i} \text{Prod.} \tag{13}$$

From the expressions above, the following equations for the steady state concentrations of the intermediates are obtained:

$$[\text{AH}-\text{HA}]_s = \frac{2 \, [E_0]}{\dfrac{k_e}{k_1 \, [\text{H}_2\text{O}_2]} + \dfrac{k_e}{k_4 \, [\text{AH}_2]}} \tag{14}$$

$$[\text{AH}-\text{HA}]_s = \frac{2 \, [E_0]}{\dfrac{k'_e}{k_1} + \dfrac{k'_e \, [\text{H}_2\text{O}_2]}{k_4 \, [\text{AH}_2]}} \tag{15}$$

$$[\text{A}-\text{A}]_s = \frac{2 \, [E_0]}{\dfrac{k_i}{k_1 \, [\text{H}_2\text{O}_2]} + \dfrac{k_i}{k_4 \, [\text{AH}_2]}} \tag{16}$$

At peroxide concentrations considerably below 10^{-3} M, deviations from the linear relationship were observed for the highest enzyme concentrations. This might probably be ascribed to the fact that the consumption of peroxide becomes relatively important as compared with the total concentration, and may not longer be regarded as constant in the expressions representing the steady state levels throughout the whole range of enzyme concentrations, but represents a limiting factor. The steady state of the blue intermediate was established at a markedly slower rate in this lower concentration range. This is contrasted with the extremely rapid establishment of the steady state levels of the active enzyme-substrate complexes in the presence of a hydrogen donor.[14]

The first part of the reaction sequence represented in eqn. (13), which results in the formation of the oxidized product of recombination, A-A,

should be analogous to the formation of the blue, stable, quinoid compound cerulignone (3,5,3',5',-tetramethoxy-4,4'-diphenoquinone) from pyrogallol-1,3-dimethylether, which evidently proceeds by the dimerization of free radicals with trivalent carbon in the para-position.[15] In this case, however, the blue, quinoid intermediate could be expected to be unstable due to the absence of the stabilizing effect of the methoxy groups, and subjected to a consequent decomposition.

Important information concerning the mode of formation and elimination of the blue intermediate was expected from a study on the effect of peroxide
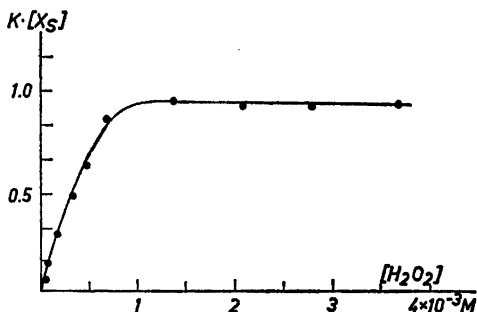

Fig. 5. The dependence of the steady state concentration of the intermediate on the peroxide concentration. HRP = 3.6×10^{-8} M. Pyrogallol = 6.3×10^{-4} M. pH = 7.0.
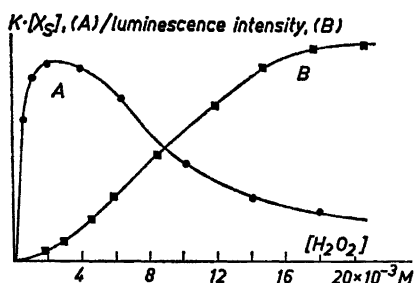
Fig. 6. The dependence of the steady state concentration of the intermediate on the peroxide concentration plotted together with the luminescence intensity measured under identical conditions. HRP = 3.6×10^{-8} M. Pyrogallol = 6.3×10^{-4} M. pH = 7.0

on the intermediate. Fig. 5 shows the effect of the concentration of oxidizing substrate on the steady state levels at a constant enzyme concentration. In Fig. 6 the same function is plotted together with the luminescence intensity over a wider range of peroxide concentrations. (The correlation of luminescence with the blue compound will be discussed in the next section). The steady state level rises steeply at first to reach a maximum, and then slowly decreases with further increase of the peroxide concentration. The presence of this optimum is, however, explained by an inhibition of the enzyme which could be observed when the overall activity was measured by recording the formation of end products at 4300 Å. This inhibition has previously been observed by early investigators of peroxidase kinetics, and was found to be reversed by the removal of excess of peroxide by catalase.[4,16] Keilin et al.[17,18] have shown a third, inactive complex of HRP to be formed in the presence of high concentrations of peroxide. Investigations by George[19] indicate compound III to be formed from compound II by the action of peroxide. In the presence of a reversible formation of the inactive complex III, the following expression for the steady state concentration of the oxidized recombination product, A-A, is obtained:

$$[A{-}A]_s = \frac{2\,[E_0]}{\dfrac{k_i}{k_4\,[AH_2]} + \dfrac{k_ik_8\,[H_2O_2]}{k_4k_9\,[AH_2]} + \dfrac{k_i}{k_1\,[H_2O_2]}} \qquad (17)$$

where k_8 is the second order rate constant for the formation of complex III, and k_9 is the rate constant for the reverse reaction. The general form of the curves represented in Figs. 5 and 6 agrees with eqn. (17) if the blue compound is identified with the quinoid intermediate in the hypothetical reaction sequence (13), but is not valid for the simple recombination product, AH-HA.
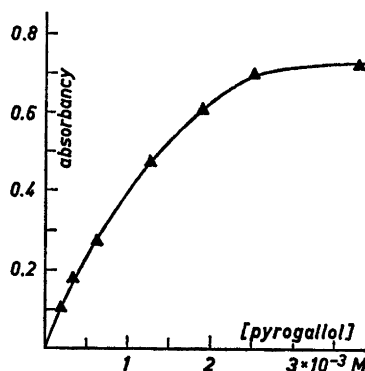

Fig. 7. The effect of reducing substrate on the concentration of the intermediate at the steady state. HRP = 3.6×10^{-8} M. $H_2O_2 = 1.75 \times 10^{-2}$ M. pH = 7.0.
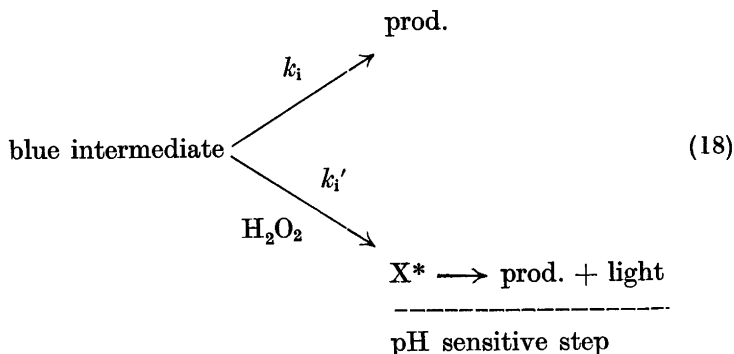
The effect of the pyrogallol concentration on the steady state is shown in Fig. 7. The levels of the blue intermediate increase with increasing concentration of reducing substrate to reach a maximum at about 3×10^{-3} M. The form of the curve will thus conform to the equations given above.

Correlation of the elimination of the blue compound and the luminescent reaction

The reaction kinetics of the primary and the secondary complexes of HRP are practically unchanged in the pH region 3.6—8.8.[14] The existence of a sharp pH optimum for the luminescent reaction is thus not directly associated with the activity of the enzyme, but reflects the presence of pH sensitive steps slower than the reaction of pyrogallol with complex II. The effect of pH on the concentration levels of the blue intermediate in the range in question was, however, found to be comparatively small. This indicates that the measurement of the steady state levels of the blue compound at 6000 Å probably gives a more reliable index of peroxidatic activity than does the conventional purpurogallin method. The formation of the strongly coloured oxidation products from pyrogallol under these experimental conditions, however, makes a more thorough investigation of this question impossible by means of the usual spectrophotometric methods.

The maximum in light intensity at pH 7.6 was not found to coincide in time with the concentration maximum of the intermediate, but is somewhat retarded as compared to the latter. This fact, as well as the relative insensitivity of the steady state levels of the blue compound to changes in pH, excludes the possibility of the elimination of the blue compound as being directly associated with the chemiluminescence. Several observations, however, indicate that the light emission accompanies a pH-dependent step immediately following a peroxide-dependent elimination. A change in the reaction velocity with the hydrogen ion concentration would explain the fact that the elimination and the luminescent reaction at pH 6.0-7.0 are telescoped into one apparently synchronous process.

The chemiluminescent reaction is extremely sensitive to changes in peroxide concentration and the luminescence is characterized by a very sharp optimum of oxidizing substrate.[3] The peroxide level required for maximum luminescence considerably exceeds the minimum concentration necessary for maximum formation velocity of free radicals. A higher luminescence intensity was found to be correlated with a lower steady state level of the blue compound which is shown in Fig. 6, i.e. whereas the activity of the enzyme is inhibited, the luminescence is greatly activated by increasing peroxide concentrations. This phenomenon is consistent with the presence of two competing mechanisms of elimination of the intermediate, only one of which being activated by peroxide and associated with luminescence. The possibility that a peroxide-dependent decomposition constitutes the luminescent reaction sequence is not surprising, in view of the fact that luminescent reactions almost without exception involve molecular oxygen or peroxides.

$$
\begin{array}{c}
\text{prod.} \\
k_i \nearrow \\
\text{blue intermediate} \\
k_i' \searrow \\
H_2O_2 \\
X^* \longrightarrow \text{prod.} + \text{light} \\
\overline{} \\
\text{pH sensitive step}
\end{array}
\qquad (18)
$$

The injection of peroxide at the steady state of the blue compound will result in a momentous activation of the luminescence which is accompanied by the rapid disappearance of the intermediate. When ethyl hydrogen peroxide was used as oxidizing substrate, pyrogallol was also found to undergo a luminescent oxidation in the presence of HRP. The blue compound formed was found to be eliminated by the addition of hydrogen peroxide in a reaction accompanied by an increased luminescence intensity in the same way as when hydrogen peroxide was used initially.

Final confirmation of the close association of the luminescent reaction and the fast, peroxide-dependent elimination of the blue compound was obtained by measuring the increase in luminesensce intensity caused by a single injection of peroxide of varying concentration at the steady state
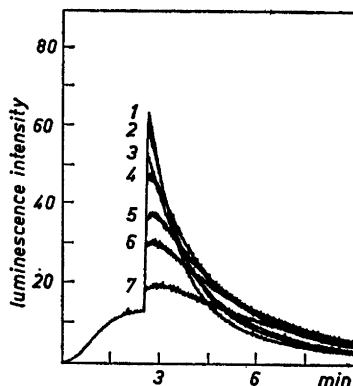

Fig. 8. The effect of injection of varying concentrations of hydrogen peroxide on the luminescence at the steady state into a reaction mixture containing: HRP = 3.6×10^{-8} M. Pyrogallol = 6.3×10^{-4} M. H_2O_2 = 7.0×10^{-3} M. Amounts of peroxide injected; 1) 22.1×10^{-5}, 2) 17.6×10^{-5}, 3) 13.2×10^{-5}, 4) 8.8×10^{-5}, 5) 6.6×10^{-5}, 6) 4.4×10^{-5}, and 7) 2.2×10^{-5} moles. pH = 7.0.

(Fig. 8). The increase in reaction velocity of the luminescent reaction is given by the sudden increase in luminescence which is obtained upon the injection of the peroxide. When these values, or alternatively, the values found by integration of the curve peaks up to a time 15 sec after the initiation of the activated luminescence, are plotted together with the relative amounts of the blue compound eliminated during this first phase of the reaction, an absolute correlation is obtained (Fig. 9). Each of the two curves are composed of two linear parts, the points of intersection of which are situated at the same peroxide concentration, 2.0×10^{-2} M. This concentration corresponds exactly to the point where a peak in the dependence of the maximal luminescence intensity in the enzymic oxidation of pyrogallol is observed when the oxidizing agent is present initially. Above this concentration, the enzymic overall activity as measured by the luminescence, becomes inhibited. This shows conclusively that the inhibition observed is not due to a decrease in elimination velocity of the intermediate blue compound, but must be localized to one of the earlier steps in the reaction sequence. Theoretically, since the position of the optimum was found to be absolutely independent of the enzyme concentration, an explanation may be offered by the formation of the catalytically inactive complex III, which then decomposes in a unimolecular reaction or in a reaction independent of the peroxide concentration. The sudden change of the elevation of the relationships of Fig. 9 probably indicates a change in elimination mechanism of the intermediate.

Pyrogallol was found to be oxidized in luminescent reactions by peroxide also when the enzyme was replaced by Fe^{3+} or Fe^{2+}. The luminescence-time dependence curves exhibited the same characteristic induction phase as when peroxidase was utilized although the molar efficiency of the inorganic ions was of course much lower. When the dependence of the reaction velocity
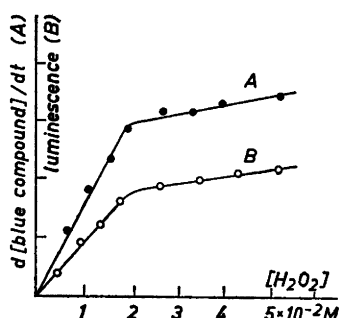
Fig. 9. The dependence of the activation of the luminescence, caused by the injection of peroxide, on the concentration of the oxidizing agent plotted together with the amount of blue compound eliminated during the initial phase of the activated elimination reaction. HRP = 3.6×10^{-8} M. Pyrogallol = 6.3×10^{-4} M. H_2O_2 initially present = 7.0×10^{-2} M. pH 7.0.
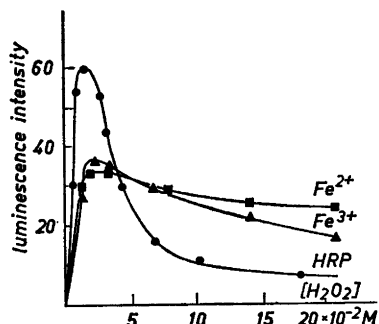
Fig. 10. The dependence of the luminescence intensity at the steady state on peroxide concentration for the enzymic reaction as well as for non-enzymic model systems containing ferrous and ferric iron. HRP = 3.6×10^{-8}. $Fe^{3+} = 7.4 \times 10^{-5}$ M. $Fe^{2+} = 7.4 \times 10^{-5}$ M. Pyrogallol = 6.3×10^{-4} M. pH = 7.0.

of the luminescent oxidation on peroxide concentration was studied in the presence of inorganic iron, a luminescence optimum, remarkably enough, was obtained at the same peroxide concentration as for the enzymic reaction (Fig. 10). It thus appears that the inhibition of the peroxidatic activity induced by high peroxide concentrations is due to a general property of ferric iron, both in peroxidase and in the free state, of becoming transformed into a catalytically inactive form by the oxidizing substrate.

The preparation of the blue compound from purpurogallin

Early investigators concerned with the elucidation of the chemical structure of purpurogallin found, that alkaline solutions of this substance would turn blue in the presence of oxygen. In order to find out if this compound is, in fact, identical with the blue compound involved as an immediate precursor in the luminescent step, the following investigation was undertaken.

To a 1 % sodium carbonate solution varying amounts of an etheral solution of analytically pure purpurogallin, containing negligible amounts of pyrogallol, were added in a series of samples. Oxygen was then bubbled through the solutions which quickly acquired a deep indigo blue colour. Willstätter and Heiss[20] have shown that, by this procedure, the purpurogallin is completely transformed into the blue pigment, and no purpurogallin will be found in the solution. When hydrogen peroxide was then added, together with a phosphate buffer of pH 7.0, the blue colour rapidly paled and the solution regained its original yellow-brown hue. This reaction was found to be accompanied by a chemiluminescence, the intensity of which was directly proportio-

nal to the amount of purpurogallin originally present in the sample. Purpurogallin alone gave no light emission under identical conditions. In the time dependence curve of the luminescence intensity, no induction phase in the luminescence was found, but the light intensity immediately reached the maximum level and then slowly decreased.

The formation of the blue compound from purpurogallin is reversible, and the purpurogallin may be regained, at least in part, by the acidification or by extensive dilution of the aqueous solution. The exact nature of the blue compound remains obscure, however, for the time being, and this matter will be more thoroughly investigated in the near future.

REFERENCES

1. Biswas, N. N. and Dhar, N. R. *Z. anorg.Chem.* **199** (1931) 400.
2. Svensnikov. B. Ya. *Compt. Rend. Acad. Sci. U.R.S.S.* **35** (1942) 278.
3. Ahnström, G., v. Ehrenstein, G. and Nilsson, R. *Acta Chem. Scand.* **15** (1961) 1417.
4. Mann, P. J. G. *Biochem. J.* **25** (1931) 918.
5. Willstätter, R. and Stoll, A. *Ann.* **416** (1918) 21.
6. Getchell, R. W. and Walton, I. H. *J. Biol. Chem.* **91** (1931) 419.
7. Willstätter, R. and Weber, H. *Ann.* **449** (1926) 156.
8. Chance, B. *J. Biol. Chem.* **151** (1943) 553.
9. Chance, B. and Maehly, A. C. in Colowick, S. V. and Kaplan, N. O. *Methods in Enzymology,* II, Academic Press, N. Y. 1955.
10. Yamazaki, I., Mason, H. S. and Piette, L. *J. Biol. Chem.* **235** (1960) 2444.
11. George, P. *Advan. Catalysis* **4** (1952) 367.
12. Chance, B. *Arch. Biochem. Biophys.* **37** (1952) 235.
13. Chance, B. *Arch. Biochem. Biophys.* **24** (1949) 389.
14. Chance, B. *Arch. Biochem. Biophys.* **22** (1949) 224.
15. Fieser, L. and Fieser, M. *Advanced Organic Chemistry,* Reinhold Publishing Corporation, N. Y. 1961, p. 767.
16. Willstätter, R. and Weber, H. *Ann.* **449** (1926) 175.
17. Keilin, D. and Mann, P. *Proc. Roy. Soc. (London)* **B 122** (1937) 119.
18. Keilin, D. and Hartree, E. F. *Biochem. J.* **49** (1951) 88.
19. George, P. *J. Biol. Chem.* **201** (1953) 427.
20. Willstätter, R. and Heiss, H. *Ann.* **433** (1923) 17.