

STUDIES ON THE MECHANISM OF THE HORSERADISH  
PEROXIDASE CATALYZED LUMINESCENT PEROXIDATION OF LUMINOLPhilip M. Prichard and Milton J. Cormier  
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An interesting feature of the peroxidation of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) by horseradish peroxidase (HRP) is that, in the presence of excess  $H_2O_2$ , light is one of the products (Dure and Cormier, 1964). We have investigated the mechanism by which light is produced in this system by stopped-flow and fluorescence polarization techniques. This is an interesting luminescent system to study in detail because it is a good model system for bioluminescent systems of the peroxidase types that have been reported previously by Cormier and Dure (1963) relative to their work on bioluminescence of Balanoglossus biminensis. This is particularly true since it was found that the kinetics of the Balanoglossid system were identical to those found in the model system.

## METHODS

Luminol was synthesized by previously described methods (Culhane & Woodward, 1964; Levy & Stephens, 1931; Drew & Pearman, 1937) and HRP was isolated and purified from fresh horseradish roots according to the method of Paul (1958). For the preparations used in these experiments the R.Z. values ranged from 2.8 to 3.0.

Cary models 14 and 15 recording spectrophotometers were used in making absorbancy measurements during titration studies and difference spectra determinations. Rapid changes in absorbancy were monitored with the stopped-flow apparatus of Gibson and Milnes (1964).

Luminescence measurements were performed using the stopped-flow apparatus

while fluorescence polarization studies were done with an instrument built by LEBACO, Inc., Boston, Mass. We wish to thank Dr. John Brewer of this department for his help in making the fluorescence polarization measurements for us.

#### RESULTS AND DISCUSSION

It is well known that the conversion of complex I to HRP requires two 1-equivalent reductions (Yonetani, 1966; Chance, 1943; Chance, 1949a; Chance, 1952; Abrams, 1949; George, 1953). We therefore examined the stoichiometry between luminol added and the amount of HRP-H<sub>2</sub>O<sub>2</sub> complex converted to free HRP. Titrations with luminol as the hydrogen donor were performed immediately after making an equimolar mixture of H<sub>2</sub>O<sub>2</sub> and HRP. The formation and disappearance of complex II was monitored at 426 mμ which is the isosbestic point for the HRP; complex I pair (Chance, 1949a; Chance, 1949b). It was found that approximately 1 mole of luminol is required for the conversion of the HRP-H<sub>2</sub>O<sub>2</sub> complexes to free HRP and when similar titrations were carried out with ferrocyanide as the hydrogen donor we found that 2 moles were required. Luminol, therefore, serves as a typical two electron donor in this system. At pH 8.0 the second order rate constant for the reaction between the HRP-H<sub>2</sub>O<sub>2</sub> complex and luminol was found to be  $2.3 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ . For the reaction between complex II and luminol the second order rate constant was calculated to be  $7.2 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ .

The fact that 1 mole of luminol is sufficient to convert 1 mole of the HRP-H<sub>2</sub>O<sub>2</sub> complex to free enzyme suggests that the lifetime of the intermediate derived from luminol must be long enough to permit reaction with complex II. It can be shown that much of this intermediate also reacts with complex I to form complex II. Electron paramagnetic resonance observations suggest that this reactive species is most likely a radical derived from luminol since preliminary observations have shown that luminol dependent radical formation does occur in the presence of HRP and H<sub>2</sub>O<sub>2</sub>.<sup>1</sup> Radicals giving smaller signals and a shorter half-life have also been found for the reaction between HRP and H<sub>2</sub>O<sub>2</sub> but are

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<sup>1</sup>We wish to thank Dr. H. Beinert for making these measurements for us. Measurements of radical signals were made by use of the rapid freezing technique of Bray (1961).

not considered important for the light reaction.

If one adjusts the HRP-luminol ratio to two to one a single turnover situation relative to light production will exist due to the rate limiting step in the conversion of complex II to free HRP. In this way an accurate comparison of the kinetics of complex II formation and disappearance with that of light production can be made. In these experiments it was found that complex II formation and light production were nearly complete within two seconds whereas conversion of complex II to free enzyme did not begin to occur for approximately three seconds. In comparing complex II formation as a function of time with that of light production it was found that the derivatives of each, at zero time, are the same within experimental error. This does not mean that the two events are concomitant and the evidence presented below indicates that they are not. In fact, light production continues for a short while after complex II formation is complete. However, light production certainly occurs prior to the production of free HRP. Under the conditions of these experiments complex III is not formed, thus eliminating it as a pathway to luminescence.

TABLE I

Effect of  $\text{H}_2\text{O}_2$  Concentration on the Quantum Yield

$\text{H}_2\text{O}_2$ (mM)	0.02	0.05	0.65	2.0	6.25
Quantum Yield ( $\times 10^3$ )	0.15	1.05	4.35	6.7	8.45

Final concentrations after mixing were as follows: HRP,  $2.5 \times 10^{-7}\text{M}$ ; Luminol,  $5.0 \times 10^{-7}\text{M}$ ; tris buffer, pH 8.0,  $1 \times 10^{-2}\text{M}$ ;  $\text{H}_2\text{O}_2$  as indicated.

Several experimental facts suggest the involvement of  $\text{H}_2\text{O}_2$  during luminescence in addition to that of forming the HRP- $\text{H}_2\text{O}_2$  complexes. For example, if the HRP- $\text{H}_2\text{O}_2$  ratio is less than one, luminescence is observed, but if this ratio is equal to or greater than one light production does not occur even though complexes I and II are formed. Furthermore, as shown in Table I, the quantum yield

of the light reaction, based on luminol, increases as a function of  $H_2O_2$  concentration. This relationship is essentially a hyperbolic one and at high  $H_2O_2$  concentrations the quantum yield approaches 0.01. These observations clearly indicate the need for the participation of  $H_2O_2$  (or something derived from it) in a secondary step leading to light emission. Thus light production occurs in a step subsequent to the formation of complex II.

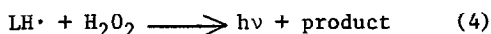
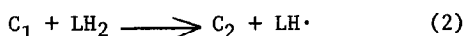
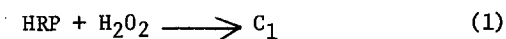
Luminol is a fluorescent compound and advantage was taken of this fact in making some direct physical measurements on the nature of complex II. If complex II is a ternary complex between complex I and luminol one should see an increase in fluorescence polarization due to the binding of luminol to complex I (Steiner & Edelhoch, 1962). Luminol was injected into the cuvette of the fluorescence polarization apparatus which contained HRP and  $H_2O_2$  ( $1 \times 10^{-6} M$ , final concentrations each). HRP plus luminol but minus  $H_2O_2$  was the control. By this procedure fluorescence polarization changes could be monitored within one second. No significant changes were detected in the fluorescence polarization of luminol during this early time period and since complex II is nearly completely formed within one second, it is clear that complex II does not contain bound luminol. A fifteen fold increase in fluorescence polarization of luminol does occur if crystalline bovine serum albumin is added to this mixture due to the binding of luminol to BSA.

When reciprocal plots of initial rate vs luminol (or  $H_2O_2$ ) concentration at different fixed concentrations of  $H_2O_2$  (or luminol) were made, normal Michaelis-Menten kinetics were observed and the lines intersect at a common point in the left hand quadrant of the plot. Secondary plots of the intercepts extrapolate through 0 for each set of data showing that the limiting maximum velocity for both luminol and  $H_2O_2$  is infinity. This suggests that the light producing step is a purely chemical one that does not directly involve participation of an enzyme complex.

A reaction scheme that satisfies the above data on the luminescent system and which at the same time is consistent with current concepts relative to the

mechanism of peroxidase action is shown in equations one through four below.

$C_1$  represents the classical ES complex between HRP and  $H_2O_2$  while  $C_2$  represents a 1-equivalent reduction of  $C_1$  by luminol ( $LH_2$ ) to produce a luminol radical ( $LH\cdot$ ). In this scheme light emission occurs as the result of the chemical interaction between  $LH\cdot$  and  $H_2O_2$ . This is undoubtedly an oversimplification. One could, for example, envision the participation of  $HO_2\cdot$  or  $OH\cdot$  radicals derived from chain propagating steps that involve  $H_2O_2$  and  $LH\cdot$ . A rate equation can be derived from this kinetic scheme that predicts our kinetic observations and which is consistent with the various direct observations on the system outlined above.



Preliminary observations suggest that 2 moles of  $LH\cdot$  rather than one is involved in the pathway to light production thus making the square root of the light intensity proportional to the concentration of  $LH\cdot$ . In fact, the calculated value of the square of the  $LH\cdot$  concentration, when plotted vs time, provides a good fit for the luminescence vs time curve when normalized to it. Apparently, therefore, square root kinetics are an integral part of the mechanism as suggested by Dure and Cormier (1964). At sufficiently high luminol concentrations, however, the square root function cancels out, on kinetic grounds, and one obtains normal Michaelis-Menten kinetics on the stopped-flow apparatus as outlined above. However, at lower luminol concentrations, where a significant fraction of the  $LH\cdot$  disappears by reactions (2) and (3), one again sees square root kinetics. Dure and Cormier (1964) observed these square root kinetics under conditions that should have resulted in a canceling out of the square root function. Those methods included much slower mixing times than was used here and must have been

a factor in their observations. The ternary complex, postulated on the basis of that data, we now know not to exist on the basis of the data presented here.

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