

Activation of Chemiluminescent Oxidations Catalyzed by Peroxidase and the Differentiation of Peroxidase Agents

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The enzymic oxidation of pyrogallol by peroxide in the presence of peroxidases is accompanied by chemiluminescence. The luminescent reaction may under certain conditions be considerably activated by addition of certain non-luminescent hydrogen donors, such as *o*-phenylenediamine. This activation is evidently due to the coupled oxidation of pyrogallol or a reaction product thereof by the primarily formed reaction products derived from the "activator". The presence of nonluminescent donor is advantageous when determining low enzyme activities; the effect observed by simultaneous oxidation of luminescent and non-luminescent donor on the luminescent oxidation of pyrogallol varies with the peroxidatic agent used, and the system may be utilized for differentiating between HRP, hemoglobin and myeloperoxidase.

The oxidation of pyrogallol has long been known to be accompanied by chemiluminescence.¹ In a previous report we have found the measurement of the light emission to be a convenient method for the study of peroxidase activity.² Light emission measurements are definitely advantageous compared to light absorption methods when determining low enzyme activities in crude preparations where impurities may cause considerable self-absorption. The mechanism of the luminescent process has been partly clarified, and the rate limiting step seems to be the production of a blue, dimeric compound from the pyrogallol radicals formed primarily. This dimeric product is then eliminated by hydrogen peroxide in a chemiluminescent process.³

Experimental: The equipment⁴ and the measurement method³ used is described elsewhere.

RESULTS AND DISCUSSION

Several other common donor substances besides pyrogallol have been tested, but only resorcinol was found to give a detectable luminescence. However, if some of these non-luminescent, reducing substrates were simulta-

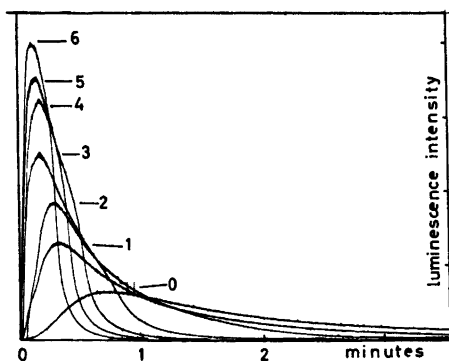


Fig. 1. Activating effect of *o*-phenylenediamine on the chemiluminescent, enzymic oxidation of pyrogallol. HRP = 3.64×10^{-9} M. Pyrogallol = 3.15×10^{-4} M. H_2O_2 = 3.5×10^{-2} M. pH = 7.0. Concentrations of *o*-phenylenediamine: 1) 0.74×10^{-5} M, 2) 1.85×10^{-5} M, 3) 3.70×10^{-5} M, 4) 5.55×10^{-5} M, 5) 7.40×10^{-5} M, 6) 11.10×10^{-5} M.

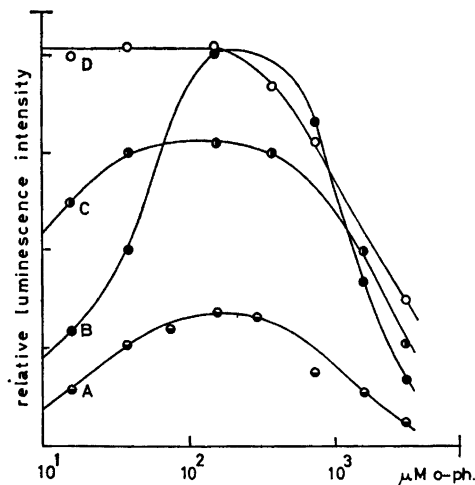


Fig. 2. Influence of *o*-phenylenediamine on the maximum "steady-state" luminescence intensity at different pyrogallol concentrations. HRP = 3.64×10^{-9} M. H_2O_2 = 3.5×10^{-2} M. pH = 7.0. Pyrogallol concentrations: A) 2.54×10^{-6} M, B) 1.27×10^{-5} M, C) 1.27×10^{-3} M, D) 2.54×10^{-3} M.

neously peroxidized together with pyrogallol, the chemiluminescence was, under certain conditions, found to be greatly activated, as shown in Fig. 1 where *o*-phenylenediamine constitutes the non-luminescent substrate. The effect of nonluminescent donor on the luminescence was found to be profoundly influenced by changes in the ratio between luminescent donor and non-luminescent donor. Fig. 2 depicts the effect of *o*-phenylenediamine on the maximal, steady-state luminescence at different levels of luminescent donor. At constant pyrogallol concentration it was found that the addition of *o*-phenylenediamine never caused an increase in the integrated luminescence yield, although the velocity of the luminescent process could be accelerated, thus giving a higher luminescence peak. At very high pyrogallol concentrations no activation could be obtained in the presence of *o*-phenylenediamine. The activation required the participation of the catalyst, since *o*-phenylenediamine by itself — neither in the pure state nor autoxidized — will affect any luminescent oxidation of the pyrogallol in the presence of peroxide.

The increase in light emission caused by the addition of *o*-phenylenediamine was found to be accompanied by an accelerated formation of the blue dimer, thus speeding up the rate limiting step. From the results obtained, it is evident that, although the reaction is accelerated by oxidation of the "activator", pyrogallol alone is responsible for the chemiluminescence. The activating effect may be explained by coupled oxidation of pyrogallol or a

product thereof by the primarily formed reaction products derived from the non-luminescent substrate. It has not been possible to correlate the activating action of *o*-phenylenediamine and other hydrogen donors *solely* to their higher reaction velocity with the rate determining enzyme-substrate complexes, as compared to pyrogallol. Other factors, such as the oxidation-reduction potentials of the primarily formed oxidation products of the activator together with steric effects, may here play an important role.

Although no absolute increase in the integrated light yield can be obtained by simultaneous oxidation of pyrogallol together with an activator like *o*-phenylenediamine, the presence of the "activator" has the important advantage of lowering to a minimum the background luminescence due to non-enzymic oxidation of pyrogallol. In this manner, the lowest amount of enzyme that could be accurately determined with our present equipment was lowered from about 5×10^{-13} to 10^{-15} moles in the sample. For determination of low enzyme activities the most satisfactory results were obtained

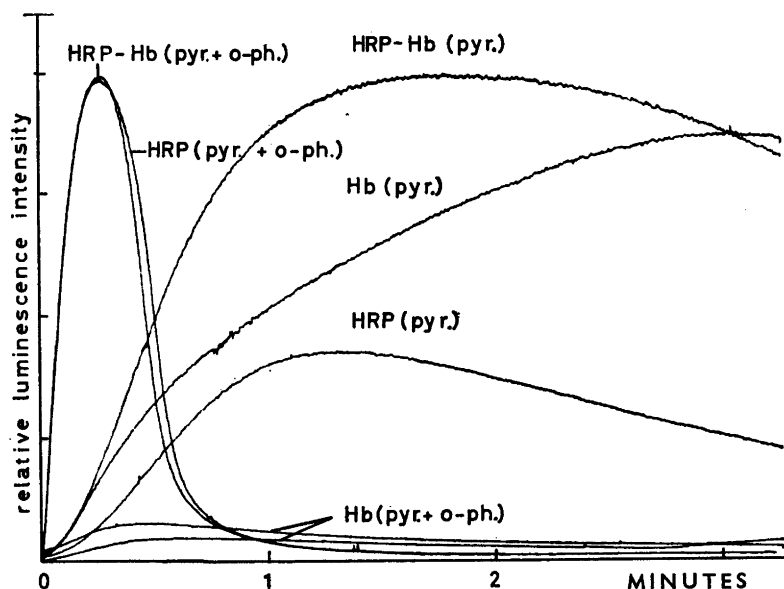


Fig. 3. Differentiation between the peroxidatic activities of HRP and hemoglobin by simultaneous oxidation of pyrogallol and *o*-phenylenediamine. HRP = 5.2×10^{-9} M. Hb = 6.2×10^{-7} M (added as hemolyzed, whole human blood). H_2O_2 = 3.5×10^{-2} M. pH = 7.0. Pyrogallol = 6.4×10^{-5} M. HRP (pyr.) = luminescent oxidation of pyrogallol induced by HRP. Hb (pyr.) = luminescent oxidation of pyrogallol induced by hemoglobin. HRP-Hb (pyr.) = luminescent oxidation of pyrogallol induced by a mixture of HRP and hemoglobin. HRP (pyr. + o-ph.) = activated oxidation of pyrogallol catalyzed by HRP in the presence of 3.73×10^{-4} M *o*-phenylenediamine. Hb (pyr. + o-ph.) = oxidation of pyrogallol catalyzed by hemoglobin in the presence of 3.73×10^{-4} M and 7.46×10^{-4} M *o*-phenylenediamine. HRP-Hb (pyr. + o-ph.) = activated oxidation of pyrogallol catalyzed by HRP and hemoglobin in the presence of 3.73×10^{-4} M *o*-phenylenediamine.

at pyrogallol concentrations ranging from 2.5×10^{-6} M to 5.0×10^{-5} M and at a concentration of *o*-phenylenediamine around 5.0×10^{-4} M.

All common methods for the assay of peroxidase activity have the disadvantage of being more or less unspecific, and a positive response will be obtained in the presence of a number of substances other than true peroxidases such as various hemoproteins and certain metal ions. Although the peroxidatic activity of HRP (horse radish peroxidase) and a contaminating hemoprotein like hemoglobin towards certain hydrogen donors may differ greatly,⁵ as a rule it has apparently not been possible to distinguish qualitatively between different peroxidatic agents. In determining the peroxidase activity of tissues the lower limit is, thus, not usually set by the sensitivity of the assay method, but is fixed by the amount of contaminating agent present having peroxidatic activity. This will also be true for the chemiluminescence measurements utilizing solely pyrogallol as reducing substrate. However, when testing peroxidatic substances with respect to luminescence enhancement caused by the presence of an "activator", quite a different situation has been observed. In contrast to the increased luminescence caused by *o*-phenylenediamine in the presence of HRP, hemoglobin-induced luminescence was found to be effectively quenched by this substance. In a model system employing HRP and whole blood it was possible to eliminate entirely the disturbing influence caused by hemoglobin which, in this case, accounted for more than 50 % of the total peroxidatic activity as given by the rate of pyrogallol oxidation in the absence of *o*-phenylenediamine, and at the same time activate the light emission caused by the action of the true peroxidase.

Preliminary investigations further indicate that, by using activated chemiluminescence determination, it is also possible to differentiate qualitatively between HRP and myeloperoxidase of leucocytes. In contrast to HRP the activity of myeloperoxidase is, under certain conditions, not influenced by the presence of *o*-phenylenediamine but is effectively activated by the presence of *p*-phenylenediamine.

Acknowledgement. The present investigation was supported by grants from *Kungl. Universitetets i Stockholm matematisk-naturvetenskapliga fakultets anslag för främjande av ograduerade forskares verksamhet, Knut and Alice Wallenberg Foundation* and the *Swedish Natural Science Research Council*. The authors also wish to express their gratitude to Dr. K. Agner for having provided us with a sample of myeloperoxidase.

REFERENCES

1. Lenard, P. and Wolf, M. *Ann. Physik und Chemie* **34** (1888) 918.
2. Ahnström, G., v. Ehrenstein, G. and Nilsson, R. *Acta Chem. Scand.* **15** (1961) 1417.
3. Nilsson, R. *Acta Chem. Scand.* **18** (1964) 389.
4. Ahnström, G. *Acta Chem. Scand.* **19** (1965) 300.
5. Aebi, H. E. *Radiation Res. Suppl.* **3** (1963) 130.

Received December 3, 1964.