Chemiluminescence of a *Cypridina* Luciferin Analogue, 2-Methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one, in the Presence of the Xanthine–Xanthine Oxidase System[1]

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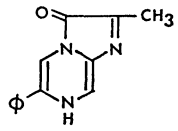
Synopsis. The title compound chemiluminesces in aqueous solutions (pH 5—6.5) in the presence of xanthine–xanthine oxidase system; O_2^- and not $^1O_2$ was proven to be the active species. Quantitative determination of xanthine and xanthine oxidase as low as 5 nmol and 10^{-5} unit, respectively, is possible by measurement of the chemiluminescence light yield and the reaction rate.

In the case of the bioluminescence of *Cypridina* system, light is emitted during the oxidation of *Cypridina* luciferin (LH) with molecular oxygen by catalysis of an enzyme, *Cypridina* luciferase. It was suggested that the substrate reacts with molecular oxygen ($^3O_2$) when it is taken in a hydrophobic pocket of the enzyme,[2] since the luciferin reacts with molecular oxygen and emits light when it is dissolved in aprotic polar solvents such as dimethyl sulfoxide, diethylene glycol dimethyl ether, *etc.*, but in aqueous solutions no luminescence is observed without the enzyme. The chemiluminescence reaction in the aprotic solvents is second order with respect to the concentrations of the substrate (LH) and molecular oxygen;[3] a suggested mechanism involves a non-chain reaction between the luciferin anion and oxygen giving a hydroperoxide anion through a radical pair intermediate:[2]

$$LH \rightarrow L^- \xrightarrow{O_2} [L\cdot\ \cdot O_2^-] \rightarrow L\text{-}OO^- \rightarrow \text{products} + h\nu.$$

Similarly its analogue, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (LA), chemiluminesces with molecular oxygen in the aprotic solvents but not in aqueous solutions.[2] It becomes chemiluminescent, however, even aqueous solutions when the reaction mixture contains some oxidants such as H_2O_2 and catalytic amounts of $K_3[Fe(CN)_6]$ or Fe^{3+}.[4] In these cases the active oxidant may be either superoxide (O_2^-) or singlet oxygen ($^1O_2$) produced by superoxide annihilation ($2\,HOO\cdot \rightarrow {}^1O_2 + H_2O_2$). To see if the superoxide can be the oxidant for the luminescence in aqueous solutions, we have examined the xanthine–xanthine oxidase (X–XOD) system since the system has been proven to produce superoxide ions.[5]



LA

Indeed, the luciferin analogue (LA) gives light in aqueous solutions in the presence of the X–XOD system (Fig. 1). The best pH range for the luminescence is between 5 and 6.5 (Fig. 2).[6] The light yield is decreased but rate of luminescence is increased as pH increases. Using 0.1 μmol of X in a 2.5 ml solution the linear relationship is obtained between the amount of LA and the total light yield up to 0.01 μmol of LA (Fig. 3). Thus, the maximum efficiency can be obtained when the molar ratio of X and LA is *ca.* 10:1.[7] When LA is present in more than 0.035 μmol, the total light yield is proportional to the amount of X ($X < 0.3$ μmol), whereas the rate of luminescence is independent of the concentration of X (Fig. 4).

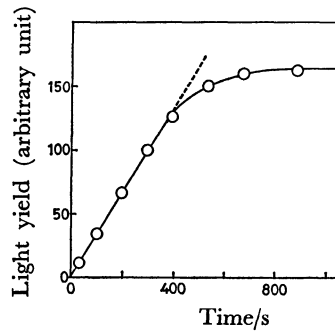


Fig. 1. Luminescence of LA in the presence of X-XOD system. X: 0.1 μmol; XOD: 0.05 unit; LA 0.01 μmol; pH 5.5.

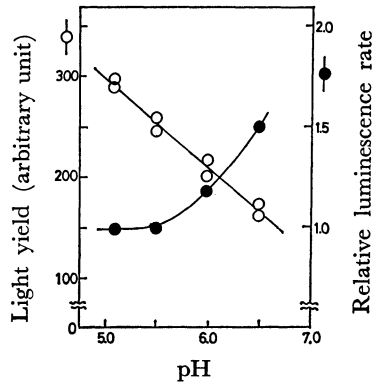


Fig. 2. pH dependence of light yield and luminescence rate. X: 0.4 μmol; XOD: 0.2 unit; LA: 0.04 μmol.

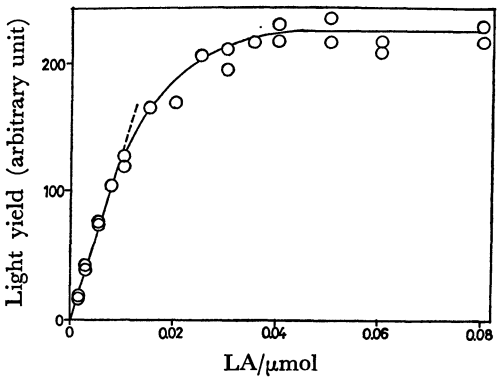


Fig. 3. Total light yield *vs.* quantity of LA. X: 0.1 μmol; XOD: 0.05 unit; pH 5.5.

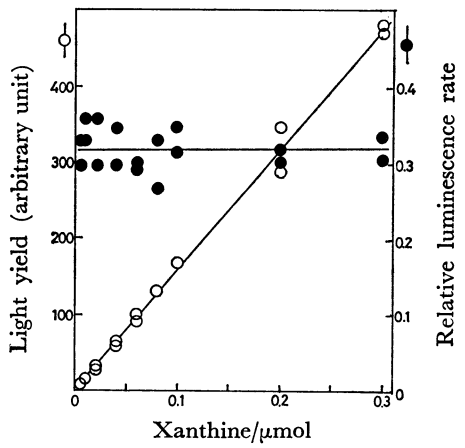


Fig. 4. Light yield and luminescence rate *vs.* quantity of xanthine. XOD: 0.05 unit; LA: 0.035 μmol; pH 5.5.

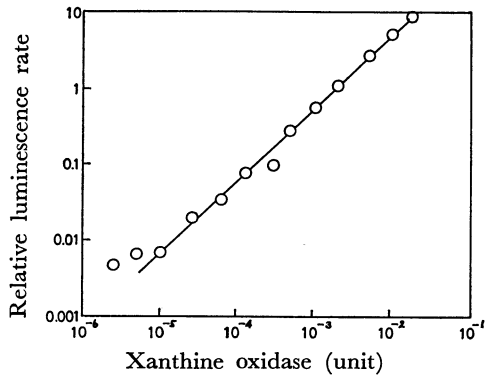


Fig. 5. Luminescence rate *vs.* quantity of xanthine oxidase. X: 0.01 μmol; LA: 0.001 μmol; pH 5.5.

Thus, the amount of X as low as 5 μmol in a 2.5 ml solution can be quantitatively determined by measuring the total light yield. On the other hand, by measurements of the initial rate of luminescence it is possible to determine the quantity of XOD as small as 10^{-5} unit (Fig. 5).

These results led to the conclusion that the reactive species of the luminescence is superoxide and not singlet oxygen;[8] if singlet oxygen were the reactive species the reaction rate must not be proportional to the amount of XOD. This was further supported by examination of the effect of sodium azide (a singlet oxygen quencher)[9] on the luminescence rate; additon of 1×10^{-3} M sodium azide did not affect the rate (4×10^{-5} M X and 0.05 unit XOD in 2.5 ml).

Involvement of superoxide was also demonstrated by addition of superoxide dismutase (SOD),[10] which is well known to decompose superoxide by the following equation: $2\cdot O_2^- + 2H^+ \xrightarrow{SOD} {}^3O_2 + H_2O_2$. SOD strongly inhibited the luminescence reaction, whereas little effect was observed with SOD preheated at 100 °C for 10 min.[11] Thus, the chemiluminescence of the luciferin analogue by the xanthine–xanthine oxidase system in aqueous solution involves superoxide as the reacting species.[12]

$$ {}^3O_2 \xrightarrow{\text{X-XOD}} \cdot O_2^- \xrightarrow{\text{LA}} h\nu $$

$$ v = k[\cdot O_2^- \text{ or } \cdot OOH] = k'[\text{XOD}] $$

The luciferin analogue may be widely applicable for detection of superoxide in physiological systems.

Experimental

Xanthine oxidase was purchased from the Boehringer Mannheim Co. The luciferin analogue (LA) was synthesized according to the literature.[13] The light yields and luminescence rates were measured with an automatic recording photomultiplier (Hamamatsu type R105)-amplifier system. Condition: 2.5 ml of 1/30 M phosphate buffer containing 10^{-4} M EDTA at 25 °C. No marked difference was observed when the buffer solution is changed from the phosphate to 0.1 M acetate.

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References

1) *Cypridina* Bioluminescence X. Preceding paper: *Tetrahedron*, **31**, 939 (1975).
2) T. Goto, *Pure Appl. Chem.*, **17**, 421 (1968).
3) T. Goto, S. Inoue, and S. Sugiura, *Tetrahedron. Lett.*, **1968**, 3873.
4) *Cypridina* luciferin does not chemiluminesce in aqueous solutions in the presence of these oxidants.[2] LA also gives chemiluminescence in aqueous solutions with KO_2+18-Crown-6 in diglyme, and with O_2+*N*,*N*′-disalicylideneethylenediaminatocobalt(II) in aqueous or methanol solutions (T. Goto, I. Kubota, N. Suzuki, Y. Kishi, and S. Inoue, "Chemiluminescence and Bioluminescence," ed by M. J. Cormier, D. M. Hercules, and J. Lee, Plenum Publ. Corp., New York (1973), p. 325).
5) I. Fridovich and P. J. Handler, *J. Biol. Chem.*, **233**, 1581 (1958); I. Fridovich, *J. Biol. Chem.*, **245**, 4053 (1970).
6) Totter *et al.* have reported that luminol and lucigenin give light in the presence of X–XOD system in strongly basic solutions (pH above 10): J. R. Totter, V. J. Medina, and J. L. Scoseria, *J. Biol. Chem.*, **235**, 238 (1960); J. R. Totter, E. C. de Dugros, and C. Riveiro, *J. Biol. Chem.*, **235**, 1839 (1960). Instead, LA gives light in physiological conditions suitable for its application to other enzyme systems.[12]
7) In the case of luminol the best results were obtained when the ratio of X and luminol is *ca.* 1:1 at pH 10.4, but the total light yield was less than one tenth of LA.
8) The X–XOD system gives extremely weak chemiluminescence; $^1O_2$ may be responsible for production of light: R. M. Arneson, *Arch. Biochem. Biophys.*, **136**, 352 (1970).
9) H. Hasty, P. B. Merkel, R. Radlich, and D. R. Kearns, *Tetrahedron Lett.*, **1972**, 49; I. Saito, K. Inoue, and T. Matsuura, *Photochem. Photobiol.*, **21**, 27 (1975).
10) J. M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969); A. J. Bard, *J. Am. Chem. Soc.*, **96**, 4023 (1974); A. P. Schaap, *J. Am. Chem. Soc.*, **96**, 4026 (1974).
11) Inhibition of chemiluminescence with SOD in the luminol (or lucigenin) +X–XOD system has been reported: L. Greenlee, I. Fridovich, and P. Handler, *Biochem.*, **1**, 779 (1962); E. K. Hidgson and I. Fridovich, *Photochem. Photobiol.*, **18**, 451 (1973).
12) The luciferin analogue also gives chemiluminescence in the system of H_2O_2–peroxidase at pH 7.5 and the system of glucose oxidase–peroxidase–O_2 at pH 5.5.
13) S. Inoue, S. Sugiura, H. Kakoi, and T. Goto, *Tetrahedron Lett.*, **1969**, 1069.