MEASUREMENT OF MICHAELIS CONSTANTS FOR ATP AND Mg^{+2} IN BIOLUMINESCENCE REACTION OF LUCIFERASE BY A HOME-MADE LUMINOMETER

Hadjmohammadi, Mohammad Reza * *and Chaichi, Mohammad Javad*

Institute of Chemistry, Mazandaran University,

P.O.Box 311, Babolsar, I.R. Iran

ABSTRACT: *Effects of ATP and Mg^{+2} concentrations on bioluminescence reaction of luciferase (Photinus pyralis) were investigated by home-made luminometer. The Michaelis constants of the enzyme for ATP and Mg^{+2} obtained from the Lineweaver-Burk graph, were 61.9 μM ± 3.3 μM and 251.6μM ± 39.0 μM respectively. Optimum concentration of Mg^{+2} for maximum luminescence was 0.004 M.*

KEY WORDS: *Michaelis constant, Luciferase, ATP, Photinus pyralis, American firefly, Luminometer, Bioluminescence*

INTRODUCTION

There are many known living organisms capable of producing light, and a famous among them is the firefly or glow-worm from *Coleoptera* order. The emission of light, is a result of chemical reaction in the lantern of firefly. The emission of light by living organism is called bioluminescence(BL). The common firefly in north America is *Photinus pyralis* [1].

The bioluminescence system of firefly which is related to click beetle of the *Pyrophorus,* was discovered by *Dubois* in 1885 [2]. He extracted some of the compounds present in firefly's lantern by hot and cold water, separately. When the materials extracted with hot water were cooled very quickly and mixed with the other, caused light emission. The materials extracted with hot and cold water are named luciferin and luciferase respectively. Luciferin in the American firefly, *Photinus pyralis*, and Japanese firefly, *Luciola,*

were discovered in 1916 and 1917 by *Harvey* respectively [3-5]. Determination of chemical structure of luciferin and it's synthesis were performed in 1961 by *White* et al. [6-7].

Identification and determination of luciferin in Iranian firefly, *Lampyris turkestanicus,* has also been reported [8]. The amino acid sequencing of luciferase in different species of fireflies were reported in 1989 [9].

Chemiluminescence reaction

Generally speaking, bioluminescence reaction is a special type of chemiluminescence (CL). These types of reactions are called cold light reactions, because the variation in temperature is low. A simple type of CL reaction is as the following:

$$A + B \longrightarrow C^* \longrightarrow h\nu(\text{light}) + D(\text{product}) \qquad (1)$$
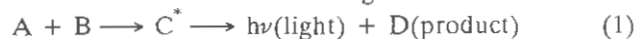
In this reaction, the reactants A and B produce

electronic excited state C^* which looses its energy by light emission through CL. The mechanism of light emission in these types of reactions are shown in Figs. 1. and 2.
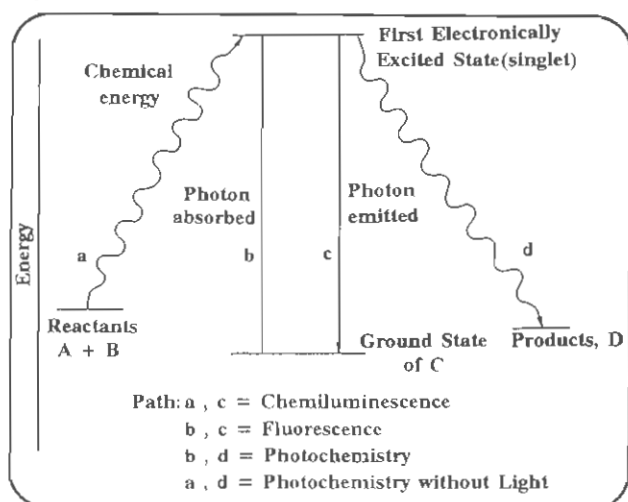

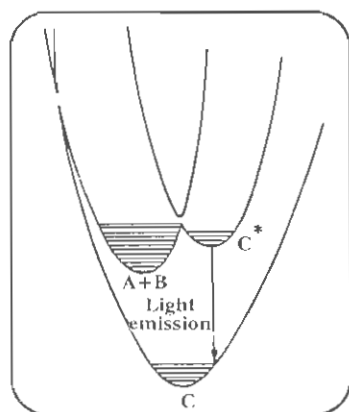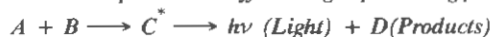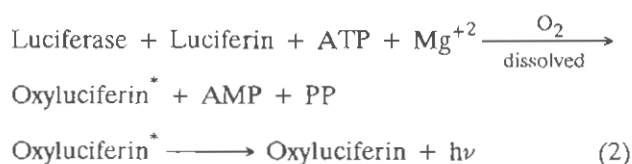Fig. 1: *Relationship between different light producing processes.*

$$A + B \longrightarrow C^* \longrightarrow h\nu \ (Light) + D(Products)$$

Fig. 2: *Formation of excited state C from reactants A and B.*

The chemiluminescence quantum yield (Q_{CL}), is defined as the ratio of the number of emitted photons to the total number of excited molecules. The Q_{CL} for firefly BL is higher than 88% [10].

Mechanism of firefly bioluminescence

The BL reaction for firefly is as follows:

Luciferase + Luciferin + ATP + Mg^{+2} $\xrightarrow[\text{dissolved}]{O_2}$

Oxyluciferin* + AMP + PP

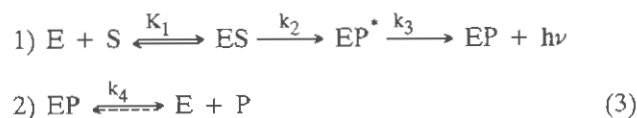Oxyluciferin* $\longrightarrow$ Oxyluciferin + $h\nu$ (2)

AMP = Adenosine monophosphate

PP = Pyrophosphate

These reactions perform in firefly lanterns in a cyclic form. The luciferase enzyme have active sites which could be occupied by luciferin, ATP, and Mg^{+2} substrates. After each emission, the enzyme is regenerated and could react again [4].

The kinetics of firefly BL reaction

For determination of *Michaelis* constant of ATP, the BL reaction is rewritten in a general form:

1) $E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP^* \xrightarrow{k_3} EP + h\nu$

2) $EP \underset{}{\overset{k_4}{\rightleftharpoons}} E + P$ (3)

where constants and terms are defined as follows: [11].

K_1 = equilibrium constant

k_2 , k_3 , k_4 = rate constants

S = substrate

E = enzyme

ES, EP, EP^* = intermediates

P = reaction product (oxyluciferin)

The reaction rate with respect to ATP is proportional to emitted maximum light intensity:

$V = I_{max} = K[ATP]_0/(K_{m,ATP} + [ATP]_0)$ (4)

where I_{max} , $[ATP]_0$, $K_{m,ATP}$ and K respectively are: maximum light intensity, original concentration of ATP, *Michaelis* constant of enzyme for ATP and a constant. K_m, *Michaelis* constant, is defined as a concentration of a substrate at which, the reaction rate is half of the maximum rate. For the case when $[ATP]_0 <<$ K_m the Eq. (4) simplifies to:

$V = I_{max} = Const. [ATP]_0$ (5)

According to the Eq. (5) luminescence intensity is a linear function of ATP concentration and its time dependence can be written as:

$dI/dt = Const. d[ATP]/dt$ (6)

The above equation provides a simple photometric means for following the variations in ATP and related compounds during BL of firefly [4,11,12].

Since the light intensity is converted to voltage in a luminometer, the Eq. (5) could be written on the basis of voltage. Generally, the use of *Michaelis-*

Menten equation, for determination of *Michaelis* constant is difficult because of the nonlinearity of light intensity vs substrate concentration. Accordingly, the reciprocal of this equation (*Lineweaver-Burk* equation) is usually used for this type of determination [11,12].

$$\frac{1}{V_0} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}} \; [\textit{Lineweaver-Burk} \text{ equation}]$$

(7)

In this equation V_{max} , is the voltage at the time when the active sites of enzyme are saturated with substrate, V_0 is the voltage at the start of the reaction when the sites are yet unsaturated case, and [S] is the concentration of substrate.

EXPERIMENTAL
Chemicals

Chemicals were obtained from the following sources: ATP assay mix, FL-AAM, and ATP standard, FL-AAS (Sigma Chemical Co., St. Louis, Missouri, USA); magnesium chloride (Fluka, Buchs, Switzerland).

Instrumentation
luminometry

The instrument used for measurement of light intensity is called luminometer or photometer whose general schematics is shown in scheme below:

Scheme 1

Detectors used are usually photomultipliers, photocells, phototubes or photographic plates [12,13].

Home-made luminometer

Since the commercial luminometer were not available in our department, we designed a home-made luminometer using a photocell as its detector. The schematic representation of this instrument is shown in Fig. 4. The detector was a photocell, BPY47 (Leybold, Huerth, Germany). For construction of this instrument a wooden chasis was chosen. The photocell was placed at the bottom of the chasis. A hole of about 2.0 cm in diameter were just made

above the photocell for inserting the flat bottom glass sample cell as close to photocell as possible. The chemicals were injected into sample cell through a pin-hole above it. The whole system was placed in a box to protect it from stray light from the environment. The photocell was connected to a recorder (Servogor 120, Leybold, Huerth, Germany) and a microvoltmeter (Leybold) via proper switch. The light intensity-time curves were recorded and the voltage was read from microvoltmeter.
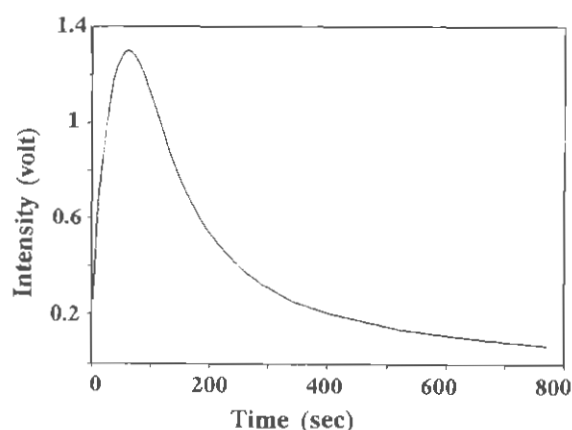
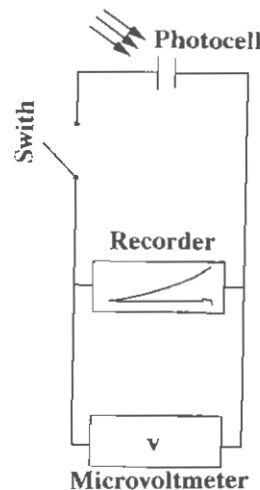*Fig. 3: Bioluminescence intensity versus time.*

Fig. 4: Schematic diagram of the home-made luminometer.

Procedure
1) Solutions
a: ATP assay solutions

5 mL of double distilled water was added to a mixture of ATP assay mix (FL-AAM), which is a lyophilized powder containing: American firefly, *Photinus pyralis* luciferase, D-luciferin, $MgSO_4$, enzyme sta-

bilizeres and tricine buffer held in an ampoule, and shaken to get a clear solution.
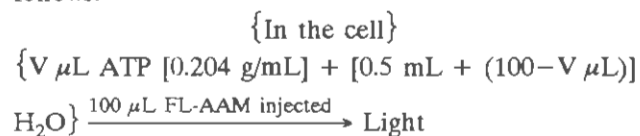
b: ATP standard solution

This was prepared by addition of 5 mL double distilled water to an ampoule containing 1.02 g of powder of ATP (FL-AAS).

2) Effect of ATP on light emission

For removal of chemical interferences (specially ATP) from the glass cells, they were soaked in 1 M HCl overnight, washed with double distilled water and dried in an oven [14].

To measure the *Michaelis* constant of enzyme for ATP substrate, the needed solutions were mixed as follows:

{In the cell}

{V μL ATP [0.204 g/mL] + [0.5 mL + (100−V μL)]

H$_2$O} $\xrightarrow{100 \; \mu L \; FL\text{-}AAM \; injected}$ Light

The time at which the ATP assay solution (FL-AAM) was injected is considered as the zero time and the voltage was measured at different times after injection. The $K_{m,ATP}$ was calculated from *Lineweaver-Burk* equation using the maximum light intensity at the beginning of the reaction.

3) Effect of Mg^{+2} on light emission

The *Michaelis* constant of luciferase enzyme for Mg^{+2} substrate, was obtained from the light emitted from the following mixture.

{In the cell}

{0.5 mL Mg^{+2} solution + 100 μL FL-AAS}

$\xrightarrow{100 \; \mu L \; FL\text{-}AAM \; injected}$ Light
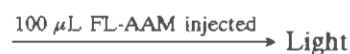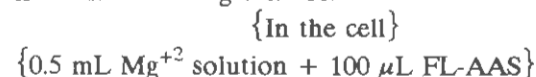
RESULTS AND DISCUSSION

The light intensity-time curve from the detector is shown in Fig. 3. This curve shows that the intensity of light is maximum after a specific time. The maximum light intensity (or related voltage) against the reciprocal concentration of ATP (*Lineweaver-Burk* curve) was linear as shown in Fig. 5. The *Michaelis* constant derived from this line was 61.9 μM±3.3 μM. The *Michaelis* constant derived by this method is in the same range as reported by other methods [15-19].
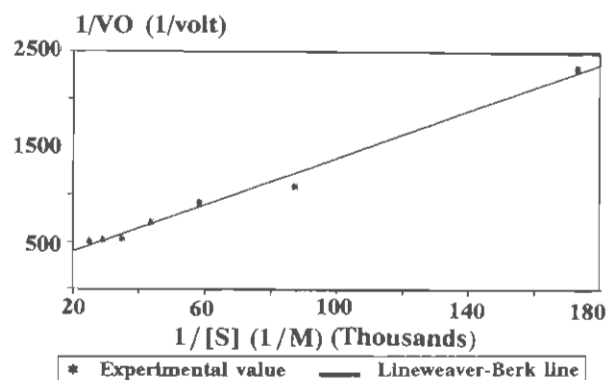

Fig. 5: Effect of ATP concentration on bioluminescence. Lineweaver-Burk graph.

Generally, The *Michaelis* constant for ATP depends on experimental conditions and concentration of reagents.

The *Lineweaver-Burk* line for ATP had a correlation coefficient of r= 0.9942 which confirms ATP as a real substrate for luciferase enzyme. Similarly, the *Michaelis* constant for Mg^{+2} substrate was found to be 251.6 μM±39.0 μM.

The optimum concentration of Mg^{+2} which corresponds to the maximum voltage (maximum light emission) was 0.004 M (Fig. 6). Since the decreasing part of the intensity-time curve for Mg^{+2} and ATP is exponential (Fig. 3), the reaction is considered first order and could be described by the following equation:

$$Ln \; V = kt + Const. \tag{8}$$

The rate constant for decreasing light intensity at optimum concentration of Mg^{2+} (0.004 M) derived from Fig. 7 and Eq. (8) was $(2.2\pm0.9)\times10^{-2}$ sec^{-1}.
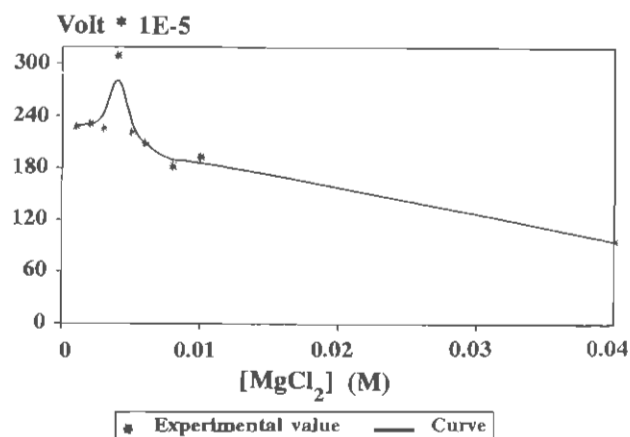

Fig. 6: Maximum voltage versus Mg^{+2} concentration.

The high concentration of Mg^{+2} inhibits the activity of the enzyme. This might be due to increasing of ionic strength and existence of Cl^- [1]. The results presented show that the bioluminescence methods based on ATP assay are sensitive and have a widespread use in the studies of biological systems [20-22]. Experimental data in this work and it's agreement with previous works show that the home-made luminometer, could be used for measurement of chemi- and bioluminescence light intensity as well as determination of rate constants in kinetic investigations.
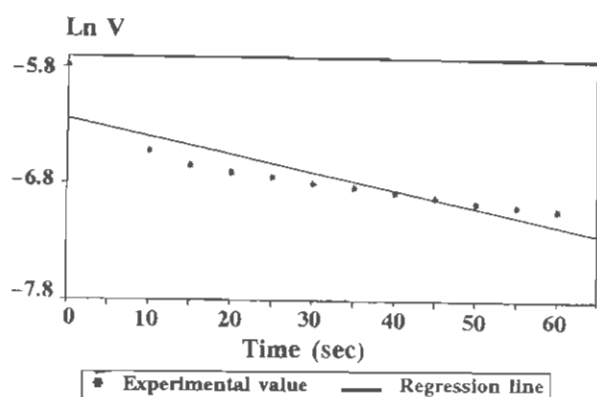

Fig. 7: Ln voltage versus time at optimum concentration of Mg^{+2} (0.004 M).

REFERENCES

[1] Macdermott, F. A., "The Fireflies of Delaware, Society of Natural History of Delaware", Wilmington, Del. (1958).

[2] Dubois, R., *C. R. Seances Soc. Biol.,* (Ser 8), **4**, 454(1887).

[3] Harvey, R., "Bioluminescence", Academic Press, Inc., New York, N.Y. (1952).

[4] Leach, F. R., *J. Applied. Biochemistery.,* **3**, 437 (1981).

[5] Goto, T. and Kishi, Y., *Angewandte Chemie.,* **7**, #6, 407(1968).

[6] White, E., McCapra, F., Field, G. F. and McElroy, W. D., *J. Amer. Chem. Soc.,* **83**, 2402 (1961).

[7] White, E., McCapra, F. and Field, G. F., *J. Amer. Chem. Soc.,* **85**, 337(1963).

[8] Hadj-Mohammadi, M. R. and Chaichi, M. J., *Photochem. and Photobiol.,* **64**, #5, 821(1996).

[9] Wood, K. V., Lam, Y. A., Seliger, H. H. and McElroy, W. D., *Science.,* **244**, 700(1989).

[10] White, E., Rapaport, E., Seliger, H. H. and Hopkins, T. A., *Bioorganic Chemistry,* **1**, 92 (1971).

[11] Ugarova, N. N., *J. Bioluminescence & Chemiluminescence.,* **4**, 406(1989).

[12] Knox, Van Pyke., "Bioluminescence & Chemiluminescence., Instrument & Applications", Vol. II, CRC Press (1985).

[13] Johnson, F. H. and Haneda, Y., "Bioluminescence in, Progress", Princeton University Press, Princeton (1966).

[14] "Sigma Technical bulletine # BAAB-1" Sigma Chem. Co. (1988).

[15] Blum, L. J. and Coulet P. R., *Biotechnology & Bioengineering.,* **28**, 1154(1986).

[16] Denbourg, L. J., Lee, R. T. and McElroy, W. D., *Arch., Biochem. Biophys.,* **134**, 381(1969).

[17] Lee, R. T., Denbourg, L. J. and McElroy, W. D., *Arch., Biochem. Biophys.,* **141**, 38(1970).

[18] Green, A. A. and McElroy, W. D., *Biochem. Biophys. Acta,* **20**, 170(1956).

[19] Webster, J. J., Chang, J. C., Monley, E. R., Spivey, H. O. and Leach, F. R., *Anal. Biochem.,* **106**, 7(1980).

[20] Campbell, A. K., "Chemiluminescence", VCH (1988).

[21] Burr, J. G., "Chemi and Bioluminescence", Dekker (1985).

[22] Leach, F. R., "Ground Water Polution Microbiology", John Wiley & Sons (1984).