Metal ion mediated inhibition of firefly bioluminescence:
A possibility via a quaternary complex

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D(-) Luciferin, interacts with different metal ions to produce colourless soluble salts with absorption spectra broader, intense and red shifted as compared to those of the parent compound. The equilibrium constants for the luciferin-metal ion system vary in the order, depository divalent transition metal ions > alkali metal ions. The equilibrium constants for the ternary complexes formed between metal ions and a mixture of luciferin and luciferase are larger than that of binary complexes but vary in the same order. Steady state fluorometric titration's of luciferin further confirmed its complexation with metal ions. The single absorption maximum of firefly luciferase at 278 nm originating from tyrosine was split into a doublet in presence of transition metal ions. The absorption maximum at lower wavelength is attributed to the H-bond raptured free tyrosine denatured conformation of the luciferase while the longer wavelength band to tyrosine-transition metal ion complex. Difference spectra of luciferase metal ion complex yielded change in the molar extinction coefficients from which the number of tyrosine molecules exposed to aqueous solution by the perturbant metal ions are evaluated following the Donovan model. The number of tyrosine molecules exposed to the aqueous medium as a result of conformational change in the enzyme are 4, 3, 2 and 3 by Hg^2+, Mn^2+, Co^2+, Cd^2+ and Cs^2+ respectively. The denaturation constants calculated for the luciferase-metal ion complexes vary between 0.152 and 0.570 and follow the order of Hg^2+>Cs^2+>Cd^2+>Co^2+>Mn^2+. Steady state fluorescence data reveal that the metal ions quench the fluorescence of enzyme by complexation with the side chain residues of the excited state tyrosine. Profound change in the UV CD spectrum of luciferin and luciferase in presence of metal ions was attributed to the conformational change in the substrate and enzyme. Thus the inhibition of luciferase activity in the firefly bioluminescence by metal ions is attributed to the quaternary complex formed between metal ion-luciferin-luciferase and ATP near or around the active site of the enzyme.

The role of metal ions on the chemical reactions has been well studied1. However, the role of metal ions on enzyme catalyzed reactions is more difficult2 to evaluate. Firefly luciferase (LA) is a 62-kDa oxygenase enzyme embedded in liposomes. It catalyses the conversion of chemical energy into light energy, during which the enzyme undergoes a large conformational change, wherein 300 of the peptide amide protons become more slowly exchangeable, making its activity and stability sensitive to buffer composition3. Optimum pH for maximum light output is 7.5 to 7.8. The firefly bioluminescence (BL) catalysed by LA occurs in two stages4, i.e., the reversible rate limiting step and the irreversible step:

\[
\text{LA} + \text{LN} + \text{ATP} \rightleftharpoons \text{Me}^{2+} \rightarrow \text{LA} \cdot \text{LN} - \text{AMP} + \text{PP}. \ldots (1)
\]

\[
\text{LA} \cdot \text{LN} - \text{AMP} \rightarrow \text{LA} + \text{AMP} + \text{OLN} + \text{CO}_2 + \text{h} \ldots (2)
\]

where PP is pyrophosphate.

It was observed that at alkaline pH, monophotonic green light emission at 562 nm occurs. On increasing the acidity to pH 6.3, biphotonic reddish green light emission at 620 nm and 562 nm occurs. On further increase in the hydrogen ion concentration, reaching a pH < 5.4, only a monophotonic light emission at 616 nm occurs5 with a profound decrease in quantum yield. A similar trend was also observed with increase in temperature6. Kajiyama and Nakona7 observed that change in a single amino acid substantially alter the emission maximum. Maimov et al.5 found that substitution of serine 286 with leucine shifted the emission maximum from 582 nm to 621 nm in Luciola migrellica LA.

Many organic molecules like benzothiazole and its derivatives9, luciferyladenylate10 and mercurobenzoates11 inhibited the activity of LA. McElroy and coworkers12 suggested that the ATP Mg^2+ is also a substrate for the enzyme action in firefly BL. The ATP binding site in LA was amply demonstrated by Lee et al.13. However, its analogue nucleotide bases such as guanine, thymine and cytosine, nucleosides and mono, di and triphosphates distort the active site
of LA and inhibit the activity of the enzyme. The inhibition of these ATP related compounds vary in the order nucleotides > nucleosides > bases. OLN, the product of the substrate LN is also a potential inhibitor of the firefly LA activity. The activity of firefly LA was inhibited by various anions. Denburg et al.\textsuperscript{14} found that the inhibition by a few anions are in the order \( \text{SCN} > \text{I} = \text{NO}_3^- > \text{Br}^- > \text{Cl}^- \) and the observed trend was traced to their position in the Hofmeister series. They further explained that a small-localized conformational change occurs in the area of the active site, upon binding to the anion, leading to inhibition of LA. In addition, pyrophosphate\textsuperscript{15} and different cations\textsuperscript{16} also inhibited the efficiency of the firefly BL. Buffers containing heavy metal ions inhibit the activity of enzymes containing sulphydryls and aromatic amino acids by specific interaction than the buffers of organic nature under optimal hydrogen ion concentration. When arsenate buffer was used and the acceptable pH range maintained, the \textit{in vitro} BL of LA was substantially quenched\textsuperscript{13}. This led to the idea of specific interaction of arsenic and other metals with LA. Subsequent studies revealed that the metal ions belonging to group IA, IIA, IB, IIB, VIIB and VIII inhibited the activity of firefly LA\textsuperscript{16,17}. While the observation of decreased efficiency in the system by metal ions cannot be contradicted, no definite explanation was found in literature except conformational change in the enzyme resulting in its inhibition.

The components LA and ATP in the rate limiting eqn.1 have competing affinity to metal ions. The linear inhibition of the activity of LA with increased metal ion concentration could be the cumulative effect of the following three factors: First, the metal ions and ATP interact strongly, with an equilibrium constant \( (K) \) varying between \( 10^6 \) to \( 10^{14} \) dm\(^3\) mol\(^{-1}\) (ref. 18-22). Large \( K \) values indicate that metal ions and ATP form stable complexes even in the presence of magnesium ions, leading to decreased luciferyl-AMP formation, resulting in overall decrease of the light output. Second, metal ions may interact with LA to alter its activity. Last, apart from the metal ions affinity to LA and ATP, they may also interact with the substrate LN, a heterocycle, blocking the formation of LA-LN-AMP and ultimately leading to diminished light output. Therefore, it is considered worthwhile to investigate the interaction between D(-) LN and LA with various metal ions employing the electronic absorption, emission, circular dichroism and BL techniques. The metal ions used belong to group IA, like Na\(^+\), K\(^+\), Cs\(^+\)and a few coloured and non-coloured transition metal ions such as Mn\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\) and Hg\(^{2+}\) as their chlorides.

**Materials and Methods**

\textit{Photinus pyralis}, North American firefly LA and LN were obtained from Amersham, UK as ATP bioluminescent monitoring kit. ATP was obtained from Boehringer Mannheim. All the metal chlorides were obtained from BDH as AR grade and used without further purification. LA and LN were dissolved in 50 mM Tricine buffer \( pH \ 7.8 \), containing \( 10 \) mM \( \text{MgSO}_4 \) and \( 1 \) mM EDTA. The stock solution of ATP (5 mM), was made in 10 mM Tris HCl buffer, \( pH \ 7.5 \) and stored at \(-80^\circ \text{C}\). Metal ion salt solutions were made as \( 1 \) M stock in double distilled water and stored at \( 25^\circ \text{C}\). The working stocks were prepared on the day of use by diluting the samples to the required concentration.

Electronic absorption spectra were obtained using Hitachi UV-VIS Spectrophotometer 150-20. Different sets of experiments were carried out for various metal ion concentrations, as well as for different metal ions, at a fixed concentration of either LN, \( 4.78 \times 10^{-3} \) M or LA, \( 2.15 \times 10^{-6} \) M. The metal ion concentrations employed were more than the physiological concentrations. The optical density and equilibrium constant of the LN-metal ion complex were monitored against water in the reference path and by selecting a wavelength at which the absorbance of the complex was higher than that of free LN. Equilibrium constants for metal ion-LN complex were obtained using two different equations. The two different equations are Ketalar\textsuperscript{23} eqn. 3 and Baba-Suzuki\textsuperscript{24}:

\[
\frac{C_A C_D}{(d-d_{eq}) (C_A + C_D)} = \frac{1}{K(e_c - e_{eq}) (C_D + C_{eq}) + 1} (e_c - e_{eq})
\]

where \( C_A \) is the metal ion concentration, \( C_D \) is the concentration of LN, \( d \) is the optical density of the complex, \( d_{eq} \) is the optical density of free LN, \( e_c \) is the molar extinction coefficient of complex, \( e_{eq} \) is the molar extinction coefficient of LN and \( K \) is the equilibrium constant. A plot of \( C_A C_D/(d-d_{eq}) \) vs \( 1/(C_A + C_D) \) gave a slope of \( 1/K (e_c - e_{eq}) \) with \( 1/(e_c - e_{eq}) \) as an intercept. The data were fitted to a straight line using a program based on the method of least squares. The equilibrium constant was determined from the slope and the intercept of the straight line. The \( K \) and the molar extinction coefficient values (\( e \)) of the complexes obtained from
the above method were further verified by Baba-
Suzuki relationship.

The difference spectra of LA-metal ion, NaOH, Urea and HCl were obtained by keeping identical concentration (2.15 \times 10^6 \text{ M}) of LA in the reference path and the metal ion-LA, LA-NaOH, LA-Urea and LA-HCI respectively in the sample beam. The denaturation constant \( K_{\text{den}} \) for free LA and LA-metal ion complex was determined from the spectrophotometric data by monitoring the optical density at their respective near UV absorption maxima, assuming a reversible denaturation reaction between native and denatured LA \(^25\) eqn. 4:

\[
K_{\text{den}} = \frac{\varepsilon - \varepsilon_N}{\varepsilon_D - \varepsilon}
\]

where \( \varepsilon_N \) is the extinction coefficient for native LA (free LA), \( \varepsilon_D \) is that of denatured LA (LA-metal ion complex) obtained under the saturating concentration of respective metal ion and \( \varepsilon \) is the extinction coefficient of the complex LA-metal ion solution.

Corrected fluorescence spectra were obtained using Hitachi spectrofluorimeter 4010. Different spectra were recorded for different concentrations of the metal ions, ranging from 1 mM to 5 mM and by keeping the concentration of LN at 6.25 \times 10^{-6} \text{ M} and LA at 2.15 \times 10^{-7} \text{ M}. The LN metal ion complex was excited at 335 nm (the absorption maxima of LN) while LA-metal ion complex was exited at 280 nm. The excitation and emission bandwidth was kept at 5 nm. The Stern-Volmer quenching constants \(^{25}\) were obtained by eqn. 5:

\[
F_0 \frac{F}{F} = 1 + K_q \tau_o [Q] = 1 + K_{SV} [Q]
\]

where \( F_0 \) and \( F \) are the fluorescence intensities of LN/LA, in the absence and presence of quencher Q, \( K_q \) is the bimolecular quenching rate constant, \( \tau_o \) is the lifetime of LN in its singlet excited state (S1) in the absence of quencher (metal ion). A plot of \( F_0 F/F \) vs Q concentration resulted in a straight line with an intercept of one and the S-V quenching constant \( K_{SV} \) was obtained from the slope of the plot. The bimolecular quenching rate constant, \( K_q \), was calculated by substituting the value of \( K_{SV} \) and lifetime of LN \( (\tau_o) \) in eqn. 5. Uncertainty in \( K_q \) was \( \pm 5\% \).

Fluorescence lifetime of LN \( (\tau_o) \) was obtained by time-resolved fluorescence intensity decay measurement \(^7\), using a Photon Technology International (Western Ontario, Canada) LS100 Luminescence Spectrophotometer in analog and single photon counting modes. The flash lamp used was thyratron gated and generates nano second pulsed flashes. The plasma gas used is nitrogen gas at a vacuum of 15+ in. of Hg. Signal to noise ratio was optimized by collecting a minimum of 5000 photons in the peak channel, for the time correlated single photon counting mode. In the analog mode, the peak decay was measured till the S/N ratio is \( \geq 50 \), in the peak channel. All the experiments were carried out using a slit width of 8 nm, for excitation and emission. The sample and the scatterer were alternated after every 10% acquisition to compensate for variations in the lamp shape and drift times occurring during the period of data collection. The data were stored in a multichannel analyzer and was analysed using an IBM PC/AT computer. Instrumental artifacts were avoided by measuring the fluorescence decay of perylene, the standard fluorophore, under identical conditions and computational procedures. The weighted residuals appeared to be random and the reduced \( \chi^2 \) ratio was around 1.1 for the expected single exponential fit of the decay curve. The lifetime obtained for perylene was 4.2 ns.

Bioluminescence assay was carried out in triplicate by employing LKB luminometer 1250. In a typical assay, the concentration of LN, LA and ATP employed were 5.25 \times 10^{-4} \text{ M}, 5 \times 10^{-7} \text{ M} and 2.5 \times 10^{-8} \text{ M} respectively and the volume was made up to 0.2 ml using Tricine buffer pH 7.8. The reaction solutions were taken in polypropylene tubes and mixed using an external mixer. The readings were taken after a 5 sec delay. Different experiments were carried out by varying the concentration of metal ions from 3.5 \times 10^{-6} \text{ M} to 5 \times 10^{-6} \text{ M} and keeping the concentration of rest of the components, in the assay, constant. A control experiment was also carried out without metal ions. The maximum light output for 5 sec was taken as the peak height. Analogous Stern-Volmer plots were constructed, wherein, the ratio of peak heights of the light output in the absence of metal ions to the presence of metal ions was plotted against the metal ion concentration, at a fixed concentration of LA, LN and ATP. The BL quenching constant \( K_{SVBL} \) was obtained from the slope of the straight line.

The CD spectra were obtained using a Jasco J-20 Spectropolarimeter at 25°C. The optical path was flushed with N$_2$ and samples scanned in between 200 and 350 nm, using a quartz optical cell of 0.1 cm path length. The spectra of LN (1.25 \times 10^{-4} \text{ M}) [LA] alone
and in presence of one another (5.16 \times 10^{-7} M), LN (1.25 \times 10^{-4} M) alone and also in presence of metal ions (12 \times 10^{-3} M) were recorded using 25 mM Tricine buffer pH 7.6. CD spectra were also recorded by changing the order of mixing. Each scan was performed thrice at a scan rate of 20 nm/min. Molar ellipticities (\theta) were calculated using eqn. 6:
\[ \theta = \frac{\theta_{0r} \times 100 \times MW}{C \times l} \]  
where, MW is the molecular weight of LN/LA, C is the concentration of LN/LA, l is the path length in cm and \theta_{0r} is the observed rotation.

Results

Ground state studies

The effect of addition of varying amounts of Co^{2+} (25-125 mM) on the electronic absorption spectrum of LN in water is shown in Fig. 1. The spectral curves show an increase in the intensity of 327 nm band (\pi-\pi^* transition) of LN with increase in the concentration of Co^{2+}. The above electronic transition also underwent a bathochromic shift with increased amounts of Co^{2+} to a maximum up to 337 nm. An isobestic point around 320 nm was noticed. The appearance of an isobestic point indicates equilibrium between free and complexed LN. Similar spectra were obtained with other transition metal ions maintaining chloride as counter ion.

The effect of metal ions on the electronic absorption spectrum of LN in presence of fixed LA concentration in water was investigated. LA has no absorption at the absorption maxima of LN-metal ion complex. It has been observed that in presence of LA lower metal ion concentration causes maximum spectral changes in LN than when it alone was studied. The equilibrium constants were determined from the intensity change of the red shifted absorption maxima of the complex following Ketalar and Baba-Suzuki relationship (Fig. 2). The spectral data and equilibrium constants for various LN-metal ion systems in presence and in absence of LA given in Table 1, shows that Na^+ cannot interact when it is added to LN alone. However, in presence of LA it interacts with LN. From Table 1, it is seen that, the equilibrium constants with a given metal ion-LN system in presence of LA is always higher than that in

![Absorption spectra of LN-Co^{2+} complex in H_2O](image)

![A typical plot showing the calculation of equilibrium constant for LN-Co^{2+} complex](image)
its absence. It is also noted that the equilibrium constants are higher with transition metal ions than the alkali metal ions and vary in the order Hg$^{2+}$ > Cd$^{2+}$ > Cs$^+$ > Mn$^{2+}$ > K$^+$ > Na$^+$ > Co$^{2+}$ in presence of LA, while in the absence of LA the order is Hg$^{2+}$ > Cd$^{2+}$ > Mn$^{2+}$ > Cs$^+$ > K$^+$ > Co$^{2+}$. The molar extinction coefficients of all the LN-metal ion complexes are higher than that of LN.

In Fig. 3 the effect of Mn$^{2+}$ and Co$^{2+}$ on the absorption spectrum of LA is shown in aqueous medium. In the absence of buffer LA exhibits absorption maxima at 225 (not shown in the figure) and 278 nm. However, with the addition of either Mn$^{2+}$ (Fig. 3A) or Co$^{2+}$ (Fig. 3B) the absorption max. at 225 nm (not shown in figure) underwent a red shift and the 278 nm transition became broader and split into two peaks, one appearing at lower and the other at higher wavelength with increased intensities. The spectral shifts are metal ion dependent and are given in Table 2. It is noticed that all the transition metal ions split the 278 nm absorption maximum of the enzyme into a doublet except Cs$^+$, which only brings about hyperchromism.

In Figs. 4, 5 the difference spectra of LA in 0.01 M HCl, 0.01 M NaOH, 0.8 M urea and LA-Mn$^{2+}$ in H$_2$O

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>$\lambda_{max}$ (nm)</th>
<th>$K(M^{-1})$ Ketalar ($\varepsilon \times 10^4$, mol$^{-1}$ dm$^3$)</th>
<th>$K^*$ (M$^{-1}$), Ketalar ($\varepsilon \times 10^4$, mol$^{-1}$ dm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>327</td>
<td>$5.72 \times 10^8$ (2.16)</td>
<td>---</td>
</tr>
<tr>
<td>K$^+$</td>
<td>328</td>
<td>$7.47 \times 10^7$ (2.04)</td>
<td>---</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>330</td>
<td>$6.64 \times 10^7$ (2.54)</td>
<td>---</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>337</td>
<td>$7.10 \times 10^6$ (2.31)</td>
<td>---</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>340</td>
<td>$1.04 \times 10^8$ (3.35)</td>
<td>---</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>355</td>
<td>$8.30 \times 10^7$ (4.31)</td>
<td>---</td>
</tr>
</tbody>
</table>

* Table 1 — Spectroscopic and equilibrium constants of LN-metal ion complex in H$_2$O

<table>
<thead>
<tr>
<th>Luciferase with</th>
<th>$\Delta \varepsilon$ (mol$^{-1}$ dm$^3$)</th>
<th>Atomic radii (Å)</th>
<th>No. of Tyrosine* molecules exposed</th>
<th>$\lambda_{max}$ (nm)</th>
<th>$K^{#}_{denatur}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>227,278</td>
<td>---</td>
</tr>
<tr>
<td>NaOH (0.01 M)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>220,245,297</td>
<td>---</td>
</tr>
<tr>
<td>HCl (0.01 M)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>225,245,295</td>
<td>---</td>
</tr>
<tr>
<td>Urea (0.8 M)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>255,282(w)297(b)</td>
<td>---</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>2884</td>
<td>1.57</td>
<td>4.0 (4.12)</td>
<td>271,285</td>
<td>0.57</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>2350</td>
<td>2.67</td>
<td>3.0 (3.35)</td>
<td>277</td>
<td>0.25</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>1355</td>
<td>1.54</td>
<td>2.0 (1.99)</td>
<td>272,282</td>
<td>0.195</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>2227</td>
<td>1.28</td>
<td>3.0 (3.18)</td>
<td>272,282</td>
<td>0.167</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>2425</td>
<td>1.26</td>
<td>3.0 (3.46)</td>
<td>272,282</td>
<td>0.157</td>
</tr>
</tbody>
</table>

* The values in the parentheses are real values.
# Uncertainty in the value of $K = \pm 5\%$
of Cd\(^{2+}\) increases, the emission curve becomes broader and shows an isoemissive point at 560 nm, indicating equilibrium between free LN and the exciplex. Similar changes in the fluorescence spectra of LN with other metal ions are also observed. Without exception all the metal ions studied, quenched the fluorescence of LN. Typical plots evaluating the quenching constants, following Stern-Volmer relationship, for LN-Co\(^{2+}\) and LN-Hg\(^{2+}\) systems, are given in Fig. 7. The Stern-Volmer quenching constants and the bimolecular quenching rate constants derived from the linear Stern-Volmer plots are shown in Table 3. The \(K_{SV}\) and \(K_q\) for LN-metal ion systems vary in the order Hg\(^{2+}\)>Co\(^{2+}\)>Mn\(^{2+}\)>Cd\(^{2+}\)>Cs\(^+\). While the \(K_{SV}\) constants vary in the range 7.96 to 619.67 \(M^{-1}\), are very low with non-transition metal ions but moderate with transition metal ions.

The lifetime of the excited LN, \(\tau_0\) was determined by time resolved fluorescence decay measurements and it was found to be 4.7 ns. The lifetime of the excited LN thus obtained was substituted into eqn. 5 in order to evaluate bimolecular quenching rate constant \(K_q\). They vary between 1.70\(\times\)10\(^9\) and 132.52\(\times\)10\(^9\) \(M^{-1}\)s\(^{-1}\). The

**Emission studies**

Alterations in the UV absorption spectra of D(-) LN in the presence of metal ions led us to investigate the LN-metal ion interactions in the excited state. The effect of Cd\(^{2+}\) on the steady state fluorescence spectra of LN in H\(_2\)O is shown in Fig. 6. The emission curves of LN at 540 nm show a decrease in intensity with added Cd\(^{2+}\) without any change in the emission wavelength maximum. However, as the concentration
bimolecular quenching rate constants are larger than the magnitudes for a possible diffusion controlled reaction. Further, the larger $K_q$ values can be attributed to the formation of a non-fluorescent complex between the metal ions and LN in the ground state. Similar observation was made by Chen\textsuperscript{29} in the case of Hg\textsuperscript{2+}-N-t-BOC tryptophan. The steady state fluorescence spectra of LN was unaffected, when titrated with K\textsuperscript+ and Na\textsuperscript+, which indicates that no interaction exists between the excited state LN and K\textsuperscript+ and Na\textsuperscript+.

Firefly LA contains all the three aromatic amino acids that emit in the near UV region. Firefly LA when excited at 280 nm gave fluorescence spectra with a shoulder at 315 nm and an emission band at 330 nm (Fig. 8). It was observed that in a protein, the tryptophan residue emission displays solvent dependency and its emission maxima vary from 328 nm in hydrophobic environment to that of 348 nm in aqueous environment\textsuperscript{26}. Therefore, the shoulder at 315 nm can be attributed to the emission from tyrosine and that at 330 nm to that of tryptophan. Though the number of tryptophan residues are only two compared to 20 tyrosine residues in a 550 amino acid polypeptide LA, the relative intensity of the 330 nm band is higher than that of 315 nm. This can probably be due to the energy transfer from tyrosine to tryptophan as these have overlapping absorption maxima. In Fig. 8A, the effect of Hg\textsuperscript{2+} and in Fig. 8B, the effect of Cd\textsuperscript{2+} on the steady state fluorescence spectra of LA is shown. Both the metal ions quench the native fluorescence of LA. However, the pattern of quenching by Hg\textsuperscript{2+} and Co\textsuperscript{2+} is different from that of Cd\textsuperscript{2+}, Mn\textsuperscript{2+} and Cs\textsuperscript+. The extent of quenching of tryptophan emission at 330 nm by Hg\textsuperscript{2+} and Co\textsuperscript{2+} is more than that of quenching of tyrosine shoulder at 315 nm and at higher concentrations, no emission from tryptophan is noticed while the less intense tyrosine emission is still retained. But the presence of Cd\textsuperscript{2+}, Mn\textsuperscript{2+} and Cs\textsuperscript+ does not alter the shape of the fluorescence band of LA and both emission bands are

Table 3—Fluorescence and bioluminescence quenching constants of LN in presence of metal ion

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Fluorescence</th>
<th>Bioluminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{sv} (M^{-1})$</td>
<td>$K_q M^{-1} S^{-1} (\times 10^5)$</td>
<td>$K_1 (M^{-1})$</td>
</tr>
<tr>
<td>K\textsuperscript+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cs\textsuperscript+</td>
<td>7.96</td>
<td>1.70</td>
</tr>
<tr>
<td>Mn\textsuperscript{2+}</td>
<td>15.15</td>
<td>3.24</td>
</tr>
<tr>
<td>Co\textsuperscript{2+}</td>
<td>176.9</td>
<td>37.83</td>
</tr>
<tr>
<td>Cd\textsuperscript{2+}</td>
<td>14.22</td>
<td>3.04</td>
</tr>
<tr>
<td>Hg\textsuperscript{2+}</td>
<td>619.67</td>
<td>132.52</td>
</tr>
</tbody>
</table>

Fig. 7—Stern-Volmer quenching plots for LN-metal ion complexes in H\textsubscript{2}O (o), Co\textsuperscript{2+}-LN; (A), Hg\textsuperscript{2+}-LN.

Fig. 8—(A): Emission spectra of LA-Hg\textsuperscript{2+} in H\textsubscript{2}O \( \lambda_{ex} \) at 280 nm [(a) LA, 2.15 x 10\textsuperscript{-7} M; (b-f), LA, 2.15 x 10\textsuperscript{-7} M + HgCl\textsubscript{2}, 1.0 x 10\textsuperscript{-4} M; 1.5 x 10\textsuperscript{-4} M; 2.0 x 10\textsuperscript{-4} M; 1.0 x 10\textsuperscript{-3} M and 1.5 x 10\textsuperscript{-3} M] (B): Emission spectra of LA-Cd\textsuperscript{2+} in H\textsubscript{2}O \( \lambda_{ex} \) at 280 nm [(a) LA, 2.15 x 10\textsuperscript{-7} M; (b-d), LA, 2.15 x 10\textsuperscript{-7} M + CdCl\textsubscript{2}, 2.5 x 10\textsuperscript{-4} M; 7.5 x 10\textsuperscript{-4} M and 2.0 x 10\textsuperscript{-3} M]
equally quenched although less efficiently than with Hg$^{2+}$ and Co$^{2+}$. The extent of quenching of LA fluorescence by metal ions is evaluated by Stern-Volmer equation, monitoring the intensity of both the emission maxima, with the added metal ion. The Stern-Volmer quenching constants along with the shifted emission maxima and isoemissive points are provided in Table 4. The quenching constants $K_{sv}$ vary in between 68 and 12130 M$^{-1}$ and follow the order of Hg$^{2+}$ > Mn$^{2+}$ > Cs$^+$ > Cd$^{2+}$. In excited state, the metal ions may interact with the side chain moieties of tyrosine and tryptophan in the excited state to form exciplexes, which facilitate the non-radiative decay. Greater the interaction, higher is the non-radiative decay and larger is $K_{sv}$. Another important observation is that larger the $K_{sv}$ values, better resolved are the two emission maxima as in the case of Hg$^{2+}$ and Co$^{2+}$. In all the enzyme-metal ion systems the emission maximum at longer wavelength did not undergo any shift and occurred only at 330 nm. Further, the lower wavelength emission maximum of the enzyme, which is ascribed to excited tyrosine-metal ion exciplex, is metal ion dependent. The larger the $K_{sv}$ values, higher the blue shift, which reveals two things. First, the metal ions interact with π electron cloud of the excited tyrosine rather than the Py orbital of the oxygen of tyrosine. Second, the stronger interaction between the excited tyrosine and the metal ion prevents the energy transfer from tyrosine side chain residue to the tryptophan residue causing the reduction in the intensity from both the emission maxima, in presence of a metal ion. The appearance of isoemissive points in between 295 nm and 300 nm further confirms the existence of equilibrium between the excited enzyme metal ion via tyrosine.

**Bioluminescence**

The yellow green luminescence for the *in vitro* firefly BL at pH 7.8 in presence of various metal ions was investigated. During *in vitro* firefly BL, the time required for reaching peak height is 5 seconds. But, when varied amounts of different metal ions are added the time required to reach the peak height is delayed with decreased light output. The quenching constants obtained from the analogous S-V plots are tabulated in Table 3 and they vary between 100 to 4.5×10$^4$ M$^{-1}$. Similar to fluorescence, the alkali metal ions have little effect on BL whereas that of transition metal ions have tremendous influence.

**Circular dichroism studies**

The CD spectra of LN and LA independently, and in presence of one another along with metal ion Hg$^{2+}$ is shown in Fig. 9. Firefly LA has a highly ordered five layered αββα crystalline structure. In buffer it exhibits a negative absorption up to 205 nm (noise depective limit of tricine buffer) with a broad maximum and minimum with larger negative θ at 224 and 212 nm (assigned to nπ* transition of peptide backbone) along with a trough at 275 nm with negative θ. Thus, LA exhibits UV-CD spectrum typical of a protein containing tyrosine. Addition of Hg$^{2+}$ (2.0×10$^{-3}$ M) and Cd$^{2+}$ (8.0×10$^{-3}$ M) shifts the peak and trough to lower wavelength accompanied by decrease in negative θ. The alteration in the LA backbone CD spectrum, in presence of metal ion, maintaining the same pattern, indicates that there is a conformational change in the enzyme in presence of metal ion and Fig. 9 reveals that the change is greater with Hg$^{2+}$.

The substrate LN is also CD active, exhibiting peaks and troughs in near UV region. A broad peak at 260 nm with positive θ, a shoulder at 280 nm and a broad maxima at 340 nm, both with negative θ, followed by troughs at 277 nm and 300 nm are attributed to π-π* transitions of aromatic ring. When LA was added to LN, the CD spectra of LN in the mixture showed a slight change, i.e. the shoulder at 280 nm red shifted to 288 nm, with increase in the negative value of θ. Similar effect of LN on LA was noticed and it is in conformity with Deluca and Marsh that the ordered α-helical structure of native enzyme is destroyed upon addition of LN. However, addition of Hg$^{2+}$ ion to LN brought about substantial changes in the CD of the latter with respect to θ. The negative maximum at 280 nm with θ value -20,000 was converted to a well-defined positive absorption maxima with θ value 5000. Similarly, the minimum at 300 nm of LN red shifted to 330 nm. When Hg$^{2+}$ was added to LA alone, the negative absorption maximum of native LA at 222 nm underwent a blue shift to 218 nm with substantial change in θ values. The effect of

| Table 4 — Fluorescence data of LA-metal ion complex in H$_2$O |
|-----------------|--------|----------|------------------|
| Metal ion      | $\lambda_{\text{max}}$ (nm) | Isoemissive points (nm) | $K_{sv}$ (M$^{-1}$) |
| H$_2$O         | 315,330 | —        | —                |
| Cs$^+$         | 330    | —        | 81.42            |
| Cd$^{2+}$      | 315,330 | 300      | 68.59            |
| Mn$^{2+}$      | 315,330 | 290      | 110.46           |
| Co$^{2+}$      | 312,330 | 300      | —                |
| Hg$^{2+}$      | 311,330 | 295      | 12130            |

# Uncertainty in the value of $K_{sv} = \pm 5\%$
Hg\(^{2+}\) on the CD of the mixture of LN and LA was studied in two ways by changing the order of mixing with a view to explore possible binding priorities to one another. When LA was added to LN-Hg\(^{2+}\) mixture, substantial changes in CD of LN occurred. The LN-Hg\(^{2+}\)-LA shows all the maxima with negative \(\theta\). Interestingly, two new troughs at 245 nm and 280 nm are noticed along with a red shifted 330 nm trough of high negative \(\theta\). However, when Hg\(^{2+}\) was added to LN-LA mixture, the 260 nm peak disappeared while 288 nm shoulder shifted to 310 nm with a phase shift and the minimum at 300 nm red shifted to 335 nm. The different CD patterns obtained for the same mixture, but by differed order of mixing, indicates that the metal ion binds to either substrate or enzyme randomly. However, the large negative \(\theta\) in the former indicates that Hg\(^{2+}\) forms a stronger complex with LN in the presence of LA.

**Discussion**

Seliger and McElroy\(^{16}\) observed that the inhibition of firefly BL by temperature, acidic \(pH\) and metal ions such as Zn\(^{2+}\), Cd\(^{2+}\) and Hg\(^{2+}\) is due to conformational changes in enzyme. LA, Vallee and Ulmer\(^{33}\) attributed the inhibition of enzyme activity in the presence of metal ions to their preferential binding to the protein side-chain functions such as cysteinyl, histidyl, sulfhydryls and consequent conformational changes in the enzyme. From Fig. 1 and Table 1 it is obvious that the metal ions strongly interact with LN, structured with oxygen, nitrogen and sulfur donating sites. A similar observation was made by Ramachandran and Witcob\(^{34}\) in the case of indole and its derivatives with various metal ions. The larger equilibrium constants for transition metal ion-LN systems than that of alkali metal ion-LN systems in aqueous medium suggests that LN acts like an electron donating ligand and forms a complex with the electron deficient metal ions. Sigel\(^{1}\) observed that the electron donating ability of a ligand, with multiple donating sites with different nature, varies in the order O<N<S and the sulfur donating sites are more discriminatory than nitrogen which in turn is having higher discriminatory ability than the oxygen donating site. Among the metal ions studied, Hg\(^{2+}\) binds strongly to LN as expected, as it is often kept off the end of the ruler scale. Nevertheless, the equilibrium constants with the remaining metals show neither Irving-Williams stability sequence\(^{1}\) dependence nor to their ionization potentials. A possible reason for the noticed trend could be that all the metal ions studied in aqueous medium might not be binding to the same donating site\(^{18}\). The alkali metal ions may be preferentially binding to the carboxyl oxygen while the dipositive transition metal ions bind to either S or N atom of LN.

The magnitudes of equilibrium constants for ternary complexes are invariably higher than that of binary complexes\(^{35}\). From Table 1 it can be seen that the equilibrium constants evaluated by monitoring LN absorption band for the LN-metal ion system, in presence of enzyme are larger than that of LN-metal ion systems in water alone. The higher equilibrium constants for LN-metal ion systems in presence of enzyme may be due to the fact that LN might have
acquired a proper conformation upon binding to the enzyme, the latter facilitating enhanced complexation with metal ion.

The side chain tryptophan residues and tyrosine absorb more strongly in the near UV region. Therefore, the absorption maximum at 278 nm in LA is attributed to the \( \pi - \pi^* \) electronic transition of the side chain aromatic rings belonging to either tyrosine or tryptophan. However, the composition of amino acids in the protein is such that there are twenty residues of tyrosine residues compared to two residues of tryptophan residues in its backbone\(^36\). Hence, the 278 nm transition in LA is predominantly due to side chain tyrosine. The splitting of the near UV absorption maximum of the protein into a doublet and increase in the intensity with the addition of metal ion suggest formation of a complex between the former and latter. The appearance of a maximum at lower wavelength can probably be attributed to the conformationally altered enzyme. The rupture of hydrogen bonds associated with the phenolic groups of tyrosine, buried in the interior of the enzyme, unfold it in presence of metal ions leading to denaturation which causes a blue shift\(^37\). Once the chromophore is transferred to water, tyrosine interacts with metal ion either with its oxygen Pi electrons or aromatic \( \pi \) electrons to give a LA-metal ion complex absorbing at longer wavelengths. Thus, the lower and longer wavelength absorption maxima of the doublet are attributed to the denatured blue shift and tyrosine metal ion complex. Similar results were obtained by Morita and Yagi, where the single absorption maximum of HMM at 280 nm resulted in two absorption maxima with the addition of ATP and its analogues, which were attributed to free and complexed tyrosine residues\(^38\).

It is interesting to note that in alkaline and acidic media the \( \pi - \pi^* \) transition of the aromatic side chain occurred at 297 nm. It is possible that in alkaline solution the tyrosine residues undergo extensive ionization of phenolic hydroxyl group leading to the absorption at 297 nm. However, the absorption at 297 nm in 0.01 \( M \) HCl can be due to the absorption by tryptophan residue, once the enzyme is denatured and the tryptophan molecules are exposed from the interior structure of the enzyme. The origin of the electronic transition with an absorption maximum at \( \geq 295 \) nm in a protein that contains both tyrosine and tryptophan is complex. In the present context, in alkaline medium it can be predominantly due to ionized tyrosine as there are twenty such residues in LA. There are only two residues of tryptophan residues per mole of LA. But, tryptophan containing indole moiety has high molar extinction coefficient and therefore sensitive to any perturbation compared to a phenolic chromophore\(^39\). Hence, the transition at \( \geq 295 \) nm in the denatured LA in acidic medium, largely originates from tryptophan, which were earlier buried in the interior of the native enzyme with little or no near UV light absorption.

The difference spectra of LA-Mn\(^{2+} \) (Fig. 5) offer a very different pattern. The difference spectra of all the transition metal ion-LA systems exhibited two absorption maxima, but all of them are \( \leq 285 \) nm (Table 2). If the metal ion interacts with tryptophan moiety of the protein then the resulting complex should exhibit a maximum at least \( \geq 289 \) nm as was observed by Chen\(^31\). The location of the absorption maxima of the complex \( \geq 285 \) nm suggests that the metal ions interact primarily with the tyrosine moiety rather than tryptophan. The number of tyrosine molecules exposed from the interior of the enzyme to the solvent water, in the presence of the denaturing metal ion perturbant is estimated using Donovan model, following the changes in the molar absorbance of tyrosine. Considering an absorbance of 700 mol\(^1\) dm\(^{-1}\) per single tyrosine\(^40\), the number of tyrosine molecules exposed to the aqueous medium, with the addition of Hg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\), and Cs\(^+\) are 4, 3, 2, and 3 respectively indicating that mercuric ions perturb the enzyme to a greater extent than other metal ions.

Binding of metal ion to the enzyme may cause a change in the environment either at the active site or in a region close to the active site of the enzyme leading to its reduced activity. Therefore, the extent of change in the conformation resulting in denaturation of the enzyme with the addition of metal ions, is evaluated as denaturation constants (Table 2), assuming an equilibrium between native and denatured enzyme\(^35\) (enzyme-metal ion complex). They vary between 0.152 and 0.570 and are in the order of Hg\(^{2+}\) > Cs\(^+\) > Cd\(^{2+}\) > Co\(^{2+}\) > Mn\(^{2+}\). Cs\(^+\), an alkali metal ion belonging to group IA, has a higher denaturation constant than that of the complexes formed with all the other metal ions, except Hg\(^{2+}\). Higher the size of the transition metal ion the greater is the denaturation constant except Cd\(^{2+}\).

Fluorescence quenching titration of LN/LA and the isoeomissive points in the fluorescence spectrum indicates a definite complexation between LN/LA and
metal ions, though the Stern-Volmer quenching constants are moderate (except with Hg\(^{2+}\)) and the excited LN does not strongly interact with metal ions as in the ground state. The moderate interaction of excited LN with metal ion can be due to altered electronic distribution making LN less electron donating in its singlet excited state than in ground state, as was observed in many aromatic carboxylic acids.\(^{41}\) The steady state fluorescence data obtained for LA-metal ion systems reveal that the metal ions quench the fluorescence of enzyme by complexation with the side chain residues of tyrosine. The quenching constants evaluated from BL display a linear relationship but have greater sensitivity than those obtained by either fluorescence or absorption studies. Transition metal, alkali metal and alkaline earth metal ions strongly interact with ATP.\(^{18,22}\) Though the concentrations of metal ions employed in BL and in absorption and emission studies differ by 10 to 100 times, the higher magnitudes of equilibrium constants and bimolecular rate constants suggest that even the micro molar levels of metal ion solutions can interact with all the components of firefly BL and cause efficient inhibition of bioluminescence. Therefore, the greater sensitivity of BL thus be due to the formation of a quaternary complex in the firefly BL system with LN, LA and ATP acting as ligands. The quaternary complex, metal ion-LN-LA-ATP formed near or at the active site may be responsible for the inhibition of enzyme activity, similar to the mechanism proposed by Lin et al.\(^{42}\) for Mut-T-enzyme activation with divalent metal ions. The red shifts in the peaks and troughs in the CD spectrum of LN with added LA and metal ions indicate that it forms complexes with LA as well as with metal ions. Thus, in presence of metal ions, the formation of a quaternary complex involving metal ion-LA-LN-ATP (the initial reversible reaction, eqn. 1) leads to the inhibition of firefly BL. Further, the transition metal ions and Cs\(^+\) interact with LA alone in ground and excited state causing conformational changes via side chain tyrosine’s residues, which leads to inefficient firefly bioluminescence.

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