# EFFECT OF DIELECTRIC PROPERTIES OF MEDIA ON KINETIC PARAMETERS OF BIOLUMINESCENT REACTION

**I. E. Sukovataya and N. A. Tyulkova**

**Effect of dielectric properties of media on kinetic parameters of bioluminescent reaction of luciferase of *Vibrio harveyi* was studied with addition of organic solvents (ethanol, methanol, acetone, ethylene glycol, formamide and dimethyl sulphoxide). Small concentration of organic solvents (0.5–18% v/v) can both activate and inhibit intensity of luminescence depending on the their physical-chemical properties, higher concentrations inhibit bioluminscence in vitro. The interaction of kinetic parameters of bioluminescent reaction and dielectric permittivity of the medium $\varepsilon$ cannot be described by a unified dependence. With addition of solvents when $\varepsilon$ ranges from 74 to 82 increase of the maximum reaction rate depends on the balance between the capacity of the solvent to form hydrogen bonds and moderate hydrophobic contacts. Addition of formamide attenuates electrostatic interactions and increases the decay rate of excited emitter. High (compared to alcohols) electron-donor capacity of DMSO and acetone helps stabilize the excited intermediate of the reaction.**

Luciferases of luminous bacteria—flavin-dependent monooxigenases catalyze the oxidation reaction of the long-chain aliphatic aldehyde (RCHO) and reduced flavin-mononucleotide ($FMNH_2$) involving molecular oxygen to a respective fatty acid emitting light quanta in the visible spectrum [1]. Luciferase is a $\alpha\beta$-heterodimer and consists of two non-identical $\alpha$- and $\beta$-subunits with the molecular mass of 43 kDa and 33 kDa, respectively. The tree-dimensional structure of the enzyme was obtained at the resolution of 1.5 Å [2]. Even though the molecular mechanism of the bioluminescent reaction has not been explored in detail, the experimental results available made possible to draw a tentative kinetic layout of bacterial bioluminescence [3, 4]. According to these notions the reaction goes through formation of stable C4a-hydroperoxiflavin intermediate in the reaction of $FMNH_2$ with molecular oxygen that has been isolated and structurally characterized [5]. Further reaction of this intermediate with aldehyde separates the charges to form a cation-anion radical pair and brings forth reverse transport of electron, forming an excited emitter by chemically initiated electron-exchange luminescence (CIEEL) [6] or through dioxerane mechanism. [7]. Emission of light is followed by decay of the quaternary enzyme–substrate complex which is the limiting stage of the bioluminescent reaction [8]. When bioluminescence is initiated by the photoreduced $FMNH_2$ rapid autooxidation of $FMNH_2$ provides for one cycle of the enzyme. This makes possible to examine kinetics of one catalytic fermentative act, which for other enzymes is mostly impossible.

Effect of microambience of the enzyme on bacterial bioluminescence studied in addition of organic solvents into the reaction medium made possible to find out considerable activation of bioluminescence in vitro in the presence of small concentrations of organic solvents of different nature [9]. Received data are agree with earlier studies on this enzyme with other aldehyde substrates [10]. Effects of organic solvents on enzymes are accounted for both their direct influence on the hydrate shell and/or active center of the protein, and changes of electrostatic and hydrophobic intra- and intermolecular interactions [11, 12]. Electrostatic interactions are assigned the central role in various biological processes, including fermentative catalysis and stability of protein macromolecules [13, 14]. These interactions are theoretically sensitive to changes in the dielectric permittivity which can be varied. This work presents results on examination of the bioluminescent reaction catalyzed by the luciferase of *Vibrio harveyi*, at a modification of the dielectric properties of the medium by addition of organic solvents.

## Methods

The luciferase from *Vibrio harveyi* (strain 1212), used in the work has undergone high purification by ion-exchange chromatography [15]. Initiated by photoreduced $FMNH_2$ the bioluminescent reaction is a short flash of light with pronounced maximum and fast decay of bioluminescence; the enzyme makes, at this, one cycle. The maximum of luminescence intensity ($I_0$) specifies maximum reaction rate and concentration of the enzyme–substrate complex formed in the course of reaction. The bioluminescent reaction decays with time exponentially and is defined by the decay rate of the excited intermediate. The light emission decay constant ($k_d$) was calculated by luminescence decay from 80% to 20% of the maximum in-

Institute of Biophysics, Russian Academy of Science, Siberian Branch, Krasnoyarsk, 660036, Russia, Fax: +7 3912 443400, e-mail: biotech@ibp.krasnoyarsk.su

**Abbreviations:** DMSO is dimethyl sulphoxide, ETGL is ethylene glycol.

tensity ($k_d$): $k_d = (\ln I_{80} - \ln I_{20})/t$. The total number of quanta $Q$ proportional to the total number of molecules of the enzyme–substrate complex that has decomposed with emission was calculated as $I_o/k_d$. Measurements were carried out with a bioluminometer designed at the Institute of Biophysics (Russian Academy of Sciences, Siberian Branch) at the temperature of 25°C. Reaction parameters were recorded with 2210 (LKB-Wallas, Finland) recorder. To measure the control a reaction was carried out in the mixture of the following composition: 10 $\mu$l of $(0.07–0.13) \cdot 10^{-7}$ M luciferase of *V. harveyi*, 50 $\mu$l of $47 \cdot 10^{-6}$ M aqueous solution of tetradecanal ($C_{14}$) (Merck, Germany), 440 $\mu$l of 0.02 M phosphate buffer, of 0.5 ml of $7.6 \cdot 10^{-5}$ M aqueous solution of $FMNH_2$ (Sigma, USA) with 10 mM EDTA (Serva, USA), pH 7. In experiments the phosphate buffer was substituted for a water-organic mixture. Concentrations of solvents were expressed in volume per cent. There were three solvent types changing the dielectric permittivity of the medium ($\varepsilon$) to a different degree: (1) $\varepsilon$-decreasing solvents (ethanol, methanol, acetone, and ETGL), (2) $\varepsilon$-increasing solvent (formamide), (3) a solvent slightly (compared to the buffer solution) changing $\varepsilon$ (DMSO). Within the concentration range of organic solvents the dielectric permittivity constant of the medium varies linearly with the concentration of a respective solvent. In the case of a mixture of two solvents use was made of averaged value of dielectric permittivity constant $<\varepsilon>$ defined by the relation $<\varepsilon> = (C_1\varepsilon_1 + C_2\varepsilon_2)/100$, where $C_i$ is relative concentration of the $i$th component ($C_1 + C_2 = 100\%$), and $\varepsilon_i$ is its dielectric permittivity [16]. For the hydrophobicity use was made of $\log P$ value ($P$ is the coefficient of solvent distribution in the two-phase water-octanol system) [17]. The experimental results obtained have been statistically processed by Excel for Windows-98.

## Results

Addition of organic solvents into the reaction medium of bioluminescent reaction changes kinetic parameters of the light flash—maximum reaction rate ($I_0$), quantum yield ($Q$) and light emission decay constant ($k_d$). At small concentrations of organic solvents (from 0.5 to 18% v/v) $I_0$ increases, at high concentration the enzyme is inactivated following the threshold pattern. With $\varepsilon$ ranging from 74 to 82, i. e., both with $\varepsilon$ decreasing and increasing relative to the buffer solution, $I_0$ increases by 50–250% as compared to control, when $\varepsilon$ was modeled by addition of acetone, methanol, ethanol and formamide (Fig. 1, curves *1–3, 5*). From Fig. 1 it is apparent that in the range of $\varepsilon$ smaller than water the increase of intensity of light emission is related to hydrophobic properties of the effector added. The degree of activation decreases with increase of solvent's hydrophobicity. E. g. maximum activation—increase of luminescence intensity 3.5 times related to control—is observed in addition of methanol, while in the presence of acetone—a more hydrophobic solvent $I_0$ increase by 50% only. Meanwhile addition of formamide that increases the dielectric permittivity of the medium and is specified by the value of $\log P$ between ethanol and methanol (Table), increases the intensity less than it could be expected. Beyond the above considered range of $\varepsilon$ (74–82) the maximum reaction rate decreases and addition of acetone inhibits luciferase activity faster than alcohols. Addition of ETGL does not change luminescence intensity in the range of dielectric permittivity from 78 to 68, the luciferase loses half of its fermentative activity only at the concentration of ETGL 33% v/v, ($\varepsilon = 66$). Meanwhile addition of DMSO—low hydrophobic solvent from the series of effectors under study and slightly changing $\varepsilon$ of the medium—decreases the maximum rate of bioluminescent reaction very fast, almost linearly.

**Maximum increasing of the bioluminescence quantum yield $Q$ (%) in the various water-organic mixtures and hydrophobicity of the organic solvents ($\log P$) [15]**

| Solvent | $-\log P$ | $Q$, % |
|---|---|---|
| Ethylene glycol | 1.93 | — |
| DMSO | 1.35 | 200 |
| Formamide | 0.65 | 170 |
| Methanol | 0.74 | 370 |
| Ethanol | 0.32 | 200 |
| Aceton | 0.24 | 220 |

The behavior of bioluminescence decay rate constant ($k_d$) as $\varepsilon$ of the reaction medium varies, as well as of the maximum reaction rate cannot be described by a single curve (Fig. 2). Changes of the light emission decay constant specifying the decay rate of the excited intermediate of the bioluminescent reaction in the events DMSO, acetone and ETGL are added to the reaction mixture, follow the changes of light emission intensity. The decay constant drastically decreases with addition of DMSO and acetone, does not change with addition of ETGL. In the presence of methanol and ethanol $k_d$ starts to decrease when the dielectric permittivity is less than 74.5 and 71.5, respectively. Only with addition of formamide, that increases the dielectric permittivity of the medium, the light emission decay constant grows and reaches steady-state. I. e., with addition of DMSO and acetone the time of recorded light response of the reaction increases 4–5 times.

The quantum yield ($Q$)—total number of photons released in the course of reaction calculated from intensity and the rate constant is affected by the variation of both these reaction parameters. Basically, the quantum yield of the bioluminescent reaction changes in analogy to the maximum reaction rate actually in the same range of $\varepsilon$. I. e., the value of $Q$ does not essentially change in the presence of ETGL, but increases with addition of small methanol concentrations and to a smaller degree with addition of ethanol, acetone and formamide. The maximum degree of activation of $Q$ (%) is presented in Table. Even though with addition of DMSO the intensity of light emission drops, the quantum yield of the reaction increases considerably, it almost doubles owing to greater decrease of the light emission decay constant. Bioluminescent reaction parameters were observed to vary in the presence of DMSO in its concentration range from 0.5 to 17% v/v, when (as it was already mentioned) the dielectric permittivity of the medium varies from 78 to 76 only.

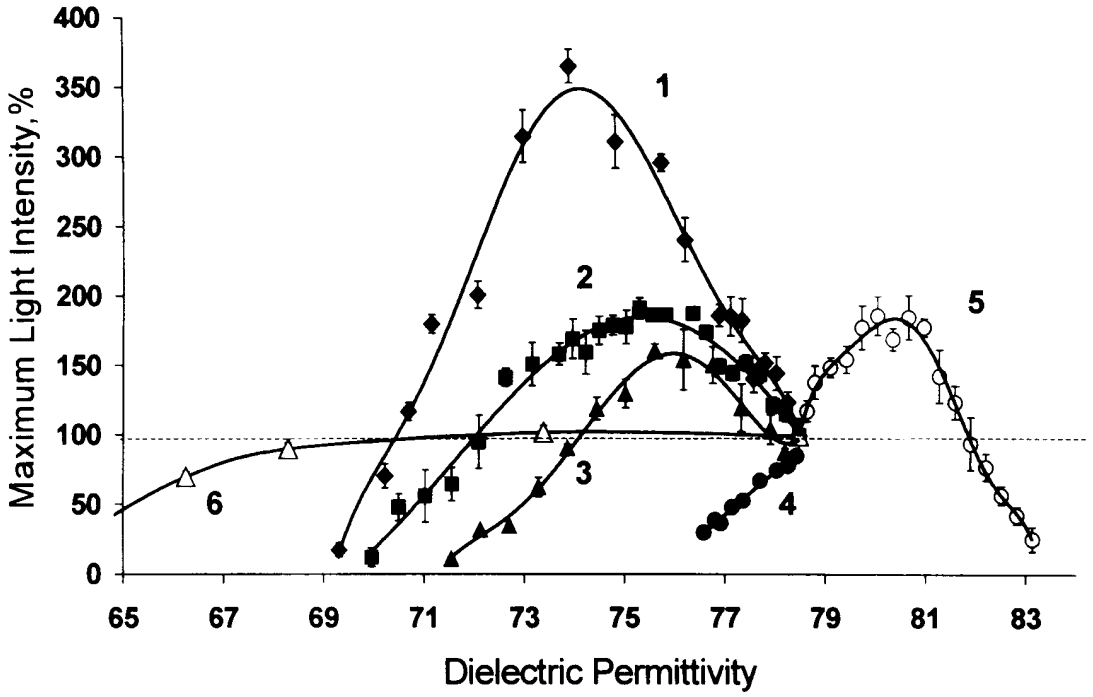


*Fig. 1.* Effect of dielectric permittivity of aqueous-organic mixtures $\varepsilon$ on luminescence intensity of bioluminescent. Maximum reaction rate $I$ (%) in the organic-solvent-free incubation medium (dash line) is taken for 100%. Organic solvents to prescribe appropriate values of $\varepsilon$ were: *1*—methanol, *2*—ethanol, *3*—acetone, *4*—DMSO, *5*—formamide, and *6*—ethylene glycol.

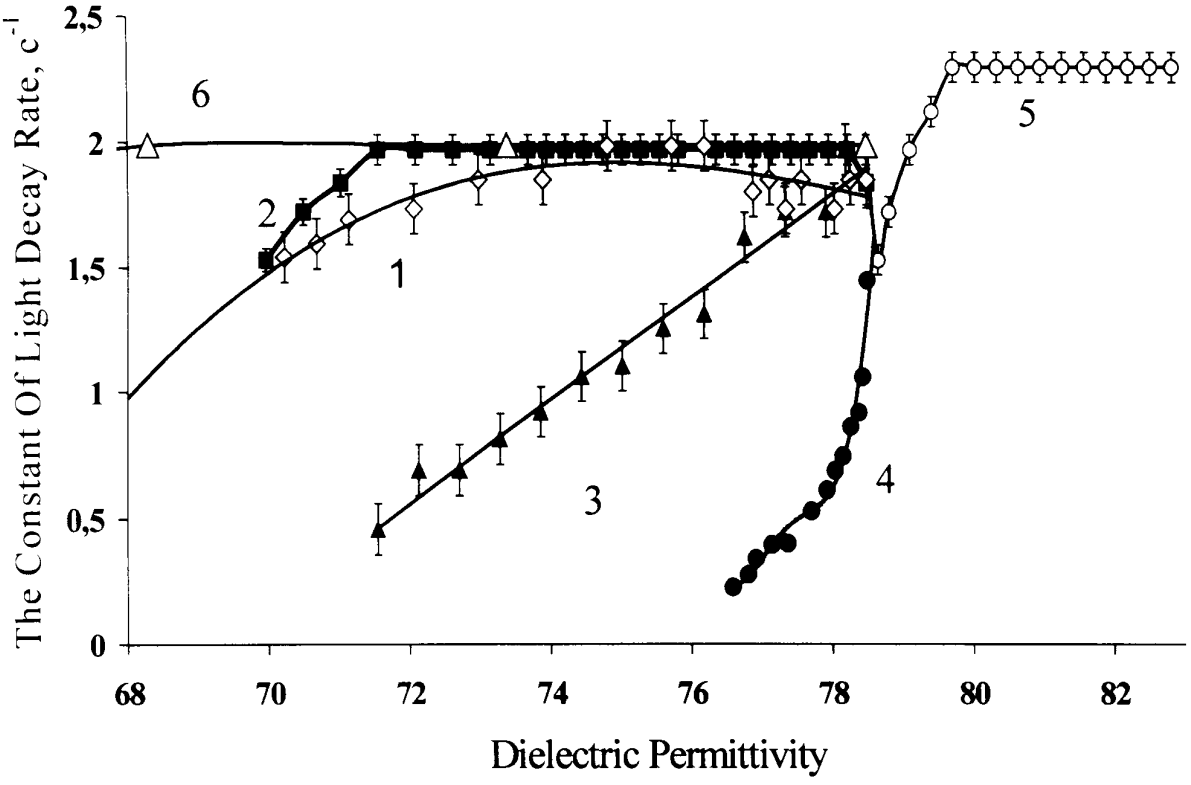


*Fig. 2.* Effect of dielectric permittivity of aqueous-organic mixtures $\varepsilon$ on the bioluminescence decay constant. Organic solvents to prescribe appropriate values of $\varepsilon$ were: *1*—methanol, *2*—ethanol, *3*—acetone, *4*—DMSO, *5*—formamide, and *6*—ethylene glycol.

## Discussion

Organic solvents are known to have manifold effect on proteins, their presence in reaction mixtures modifies not only the dielectric permittivity, but other bulk properties of the medium, too. Accordingly changes the balance of non-valent intra- and intermolecular interactions, basic among them are hydrophobic and electrostatic [11, 18–20].

From these results it is apparent that the kinetic parameters of bioluminescent reaction largely depend on the chemical nature of solvents. Decrease of $\varepsilon$ of the reaction medium in the broad range (from 78 to 67) with addition of ETGL does not bring forth essential changes of kinetic parameters of bioluminescence, i. e., stronger repulsion of similar charges and attraction of opposite charges does not seem to make a contribution into the effect of bioluminescence activation. On the other hand, in the same range of $\varepsilon$ other organic solvents bring about both very strong increase and considerable inhibition of the maximum reaction rate. Analysis of the ratio of the activation degree and the inhibition with the nature of organic solvents demonstrates that the activation is in good qualitative agreement with the capacity of organic solvents to form hydrogen bonds [21] (which also belong to the class of electrostatic interactions) with concurrent attenuation of hydrophobic contacts. E. g. methanol is a solvent less hydrophobic than ethanol and acetone, it feature higher capacity to form hydrogen bonds than ethanol and acetone who have no donors of hydrogen bonds. ETGL is a two-atom alcohol, and even though it is capable of forming hydrogen bonds, is not specific for high hydrophobicity. Electrostatic interactions attenuated by addition of formamide that forms well a volume network of hydrogen bonds and is quite hydrophobic substantially activates the bioluminescent reaction even against the background of attenuating electrostatic contacts. Minimal increase of $I_0$ is observed in acetone which turns into inhibition with addition of DMSO. Even though both these solvents belong to the class of polar aprotic solvents DMSO is a low-hydrophobic solvent. So, it is the balance of hydrogen bonds and hydrophobic contracts that provide for the maximum affinity of the enzyme and the substrate that determines the increase of bioluminescence $I_0$.

The interaction between the light emission decay constant and properties of solvents added into the reaction medium is of somewhat different nature, because $k_d$ specifies a different stage of the reaction when the enzyme–substrate has already formed. In the range of $\varepsilon$ under study the changes of the light emission decay constant depends on the dielectric properties of the medium. I. e., attenuation of electrostatic contacts with addition of formamide increases the decay rate of the excited intermediate. These results are in good agreement with the CIEEL-mechanism providing for increasing decay rate of the excited product with polarity of the medium [22]. Meanwhile, with the $\varepsilon$ of medium decreasing when the electrostatic contacts strengthen it is the donor-acceptor properties of the solvent that come to the forefront [17]. Arrangement of dependencies of the $k_d$ on $\varepsilon$ of the medium is in good qualitative agreement with their electron-donor-acceptor properties. I. e., in a dipolar aprotic solvent DMSO the light emission decay constant decreases the most, more than with addition of acetone which is a weaker electron donor. In solvents with weaker donor capacity $k_d$ of the light emission practically does not change. So, high electron donor capacity of DMSO acts as the major factor decreasing $k_d$ by higher feasibility of formation of excited emitter. This factor also defines the increase of the quantum yield of the bioluminescence. Assumingly, $Q$ of the reaction in the case of methanol, ethanol, acetone and formamide increases with concentration of the enzyme–substrate complex by additional hydrogen bonds forming with concurrent attenuation of hydrophobic contacts.

It is apparent that changes in the dielectric properties of the medium affect kinetics of bioluminescent reaction and electrostatic interaction can play not unimportant role as a factor affecting the feasibility of formation and decay rate of the excited intermediate of the reaction. However, these changes take place against the background of other, also varying characteristics of the reaction medium and adequate interpretation of varying kinetic parameters of bioluminescence requires a more detailed, and not only qualitative consideration of other specific and non-specific interactions.

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