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EXPERIMENTAL ARTICLES

Effect of Na^+ and K^+ Ions on the Luminescence of Intact *Vibrio harveyi* Cells at Different pH Values

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Abstract—The bioluminescent activity of intact *Vibrio harveyi* cells loaded with different concentrations of NaCl and KCl at different pH values was studied. In the pH range of 6.5–8.5, the effect of Na^+ was significantly higher than that of K^+ at all concentrations studied. Maximum luminescent activity was observed in cells loaded with 0.68 M NaCl. When Na^+ was nonuniformly distributed on the plasma membrane, the cell luminescence kinetics was nonstationary in the 20-min range: during incubation, the luminescence intensity increased at pH 6.5 and decreased at pH 8.5. The activation and damping rate constants depended on the Na^+ gradient value. The maximum of luminescent activity shifted during incubation from pH 8.5 to 6.5–7.0. The luminescence kinetics in the systems with KCl was stationary; the maximum level of luminescence was observed in the pH range of 7.0–7.5. Under Na^+-controlled conditions, the cell respiration and luminescence changed in synchronism. The protonophore CCP at a concentration of 20 μM completely inhibited luminescence at pH 6.5 and was ineffective at pH 8.5.

Key words: bioluminescence, monovalent cations, membrane potential, *Vibrio harveyi*.

Na^+ ions play an important role in the bioenergetics of many species of marine bacteria of the genera *Vibrio*, *Alteromonas*, and *Alcaligenes* [1, 2]. It was established that, under alkaline conditions at pH 8.5, the respiratory chain of these bacteria functions as a Na^+-pump [3–6]. The $\Delta\mu Na^+$ generated by respiration is used to perform chemical (ATP synthesis) [7] and mechanical (flagellum rotation) work [8], as well as to carry out energy-dependent metabolite transport into the cell [9].

The mechanism of $\Delta\mu Na^+$ generation has been studied in detail in the marine alkalitolerant bacterium *Vibrio alginoliticus* [3, 5–8]. In this bacterium, $\Delta\mu Na^+$ is generated by the NADH : quinone oxidoreductase (Na^+-NQR) complex of the respiratory chain. In *Vibrio costicola*, *V. parahaemolyticus*, and a number of other marine bacteria, $\Delta\mu Na^+$ is formed in a similar way [1, 4]. The involvement of $\Delta\mu Na^+$ in the conversion of chemical energy to light energy in marine luminous bacteria was for the first time considered by Wada *et al.* [10]. As distinct from other species of marine bacteria, photobacteria have a luminescence-related electron-transport system that bypasses the respiratory chain at the level of cytochromes. NADH dehydrogenase (NADH : FMN(quinone) oxidoreductase) is a common initial segment of both chains [11]. Based on the high affinity of the bioluminescence reaction of *Vibrio harveyi* in alkaline media to Na^+ ions (as compared to K^+, Li^+, and Rb^+ ions), as well as on the insensitivity of growth and luminescence to the protonophore *m*-chlorocarbonyl cyanidephenylhydrazone (CCP), Wada *et al.* [10] arrived at the conclusion that the luciferase system might be connected with the Na^+ pump. The inhibitory analysis with heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) revealed similarity between the $\Delta\mu Na^+$ generation system in *V. harveyi* and that in *V. alginolyticus*. The results gave evidence of the $\Delta\mu Na^+$ generation in photobacteria by the NADH-dehydrogenase segment of the electron-transport chain. Na^+-translocating NQR was isolated from the luminous bacterium *V. harveyi* and characterized in detail [12]. A high degree of homology of the Na^+-NQR-operon from this bacterium with that from *V. alginolyticus* was shown. The Na^+-NQR isolated from *V. harveyi* generated a Na^+-dependent transmembrane electrogenic potential in reconstructed proteoliposomes.

However, some of the data obtained by Wada *et al.* [10] were contradictory to the existence of a relationship between $\Delta\mu Na^+$ and luminescence: in acid media at pH 6.5, the effect of K^+ ions on cell luminescence was higher than that of Na^+ ions. In that work, only one (0.3 M) cation concentration in the medium and a fixed analysis time (5 min) were used. At the same time, the optimal Na^+ concentrations for different strains of photobacteria range from 0.34 to 0.68 M; the Na^+ concentration influences the emission intensity and kinetics in a complicated way (the effect depends on the pH and temperature) [13]. The involvement of $\Delta\mu Na^+$ in the process of energy transformation implies the influence of the Na^+ concentration gradient as well. It was shown [7] that the nonequilibrium distribution of ions on the plasma membrane of *V. alginolyticus* influences ATP synthesis. Under the conditions of a Na^+ ion gradient,

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P. phosphoreum cells exhibited nonstationary emission for more than 30 min [14].

To elucidate the functional role of Na^+ ions in the processes of energy transformation by luminous bacteria, we undertook a study of the kinetics and intensity of the luminescence exhibited by intact *Vibrio harveyi* cells at different pH values and different concentrations and gradients of NaCl and KCl on the plasma membrane.

MATERIALS AND METHODS

The subject of investigation was the luminous bacterium *Vibrio harveyi* B-392, ATCC provided by J. Hastings (Harvard University, United States). Cells were grown in medium of the following composition (g/l): NaCl, 30.0; Na_2HPO_4, 5.3; KH_2PO_4, 1.7; $(NH_4)_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; yeast extract, 1.0; peptone, 5.0; glycerol, 3 ml; pH 7.5.

The physiological state of the bacterial culture was monitored by the biomass increment and luminescence intensity [15]. The biomass was determined by measuring light diffusion at 660 nm on a Beckman-26 spectrophotometer (United States) and converting it to the number of cells in 1 ml of medium (N) using a calibration curve. The luminescence intensity (I) was determined using a 1251 LKB-Wallac (Sweden) luminometer calibrated by Hastings and Weber's standard [16]: 1 unit = 2×10^8 quanta/s. The luminescence was expressed in arbitrary units. Cells from the late exponential phase (7 h of growth) were precipitated by centrifugation (5500 g, 20 min) and washed twice with a buffer containing 25 mM Tris–HCl, 30 mM $MgSO_4$, and 0.26 M NaCl (pH 7.0). Washed bacterial cells were loaded with salts as described in [4, 7]. The cells were suspended in 0.26 or 0.68 M NaCl or 0.2 or 0.54 M KCl solutions and incubated at 4°C for 20 h. The luminescence was measured after introducing the cells that were loaded with different salt concentrations into 1 ml of the measurement medium (100-fold dilution, ~10^7 cells/ml) and a 5-min incubation at room temperature. To assess the specific activity, the cell concentration in the sample was determined in parallel. The measurement media were the same as that described by Wada *et al.* [10]: 25 mM Tris–HCl buffer with NaCl concentrations of 0.17, 0.34, and 0.52 M or KCl concentrations of 0.13, 0.27, and 0.4 M; the pH was fixed in the range between 6.5 and 8.5. The cell suspension introduced did not change the pH of the measurement media. The activation rate constants (k_a) at pH 6.5 were calculated according to the equation of the hyperbolic dependence in the $\ln(I_n - I_{n-1})/\Delta t$ coordinates. The damping rate constants (k_d) at pH 8.5 were calculated according to the equation of the exponential dependence in the $\ln I/t$ coordinates. The oxygen consumption was determined by Clark's closed electrode on a LP-7E polarograph.

Table 1. Specific luminescent activity (Q) of *V. harveyi* cells at different KCl and NaCl gradients in 25 mM Tris–HCl buffer, pH 7.0

[KCl] intracellular/extracellular, M	Q, quanta /(s cells)	[NaCl] intracellular/extracellular, M	Q, quanta /(s cells)
0.2/0.13	2 ± 2	0.26/0.17	1369 ± 60
0.2/0.27	4 ± 2	0.26/0.34	1321 ± 50
0.2/0.4	3 ± 2	0.24/0.52	1000 ± 30
0.54/0.13	3 ± 2	0.68/0.17	4140 ± 100
0.54/0.27	248 ± 30	0.68/0.34	4053 ± 50
0.54/0.4	753 ± 150	0.68/0.52	3054 ± 10

RESULTS

The comparative analysis of the efficiency of monovalent cations was performed on intact bacterial cells preloaded with different NaCl and KCl concentrations and in media with different concentrations of these ions.

Table 1 shows the values of specific bioluminescent activity (quanta/(s cells)) at pH 7.0 after a 5-min incubation of the cells. Cells loaded with 0.2 M KCl virtually did not emit light, irrespective of the extracellular KCl concentration. A low-level emission was also observed with cells loaded at a 0.54 M KCl at a 0.13 M KCl concentration in the incubation medium. The loss of luminescence was reversible and could be restored by increasing the concentration of extracellular KCl. When extracellular KCl concentration was increased to 0.4 M, the emission activity increased by more than 100-fold; however, in no case did the luminescence with KCl exceed the values recorded with NaCl. The specific activity of cells loaded with 0.26 M NaCl was almost one thousand times higher than that of cells loaded with 0.2 M KCl and changed slightly with the extracellular NaCl concentration. Cells loaded with 0.68 M NaCl were characterized by the highest bioluminescent activity, which varied insignificantly with the extracellular NaCl concentration in the 0.17–0.52 M range. Thus, the results obtained show that at pH 7.0, the emission activity of cells is significantly higher with Na^+ than with K^+ ioins and does not significantly depend on the intracellular NaCl concentration in the 0.26–0.68 M range if the analysis time is within 5 min.

The analysis of the pH dependence of the luminescence activity, which was performed using artificially created ion gradient, demonstrated the nonstationary nature of the luminescence kinetics during a 20-min cell incubation in both acid and alkaline media: at all of the extracellular Na^+ concentrations used, luminescence activation was observed during incubation at pH 6.5 and luminescence damping at pH 8.5. The luminescence damping rate at pH 8.5 was significantly higher than the luminescence activation rate at pH 6.5.

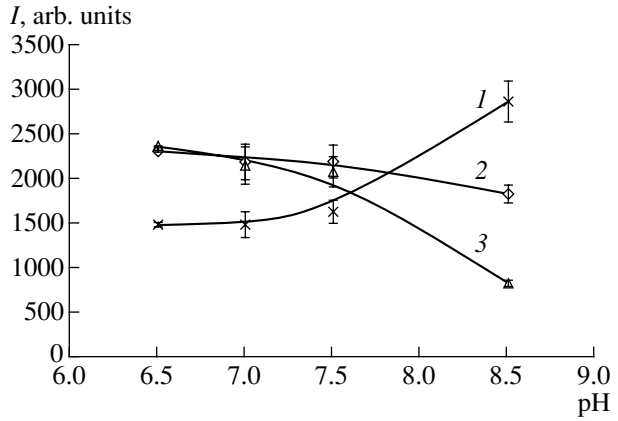

Fig. 1. pH dependence of the luminescence (*I*, arbitrary units) of *V. harveyi* cells loaded with 0.68 M NaCl after (*1*) 9, (*2*) 16, and (*3*) 24 min of incubation in 25 mM Tris–HCl buffer containing 0.52 M NaCl.

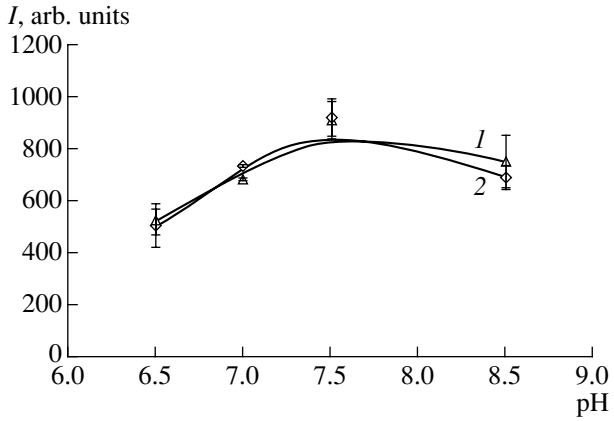

Fig. 2. pH dependence of the luminescence (*I*, arbitrary units) of cells loaded with 0.26 M NaCl after (*1*) 5 and (*2*) 20 min of incubation in 25 mM Tris–HCl buffer containing 0.26 M NaCl.

The consequence of this is a change in the profile of the pH dependence of luminescence with time.

Figure 1 shows the pH dependencies of the luminescence intensity (*I*, arbitrary units) of cells loaded with 0.68 M NaCl after 9, 16, and 24 min of incubation in medium with 0.52 M of NaCl. After 9 min of incubation, the maximum luminescence level was observed in medium with pH 8.5. In the course of further incubation, maximum luminescence was observed at pH 6.5. A similar picture was observed at a NaCl concentration of 0.17 M in the medium: maximum luminescence at pH 8.5 at the beginning of the experiment and at pH 6.5 after 20 min of incubation. A detailed study of the kinetics showed that temporal dependencies of the luminescence activation and damping are well described by the equations of hyperbolic (pH 6.5) and exponential (8.5) dependence, with the coefficients of correlation (r_i) for different curves varying between 0.85 and 0.95. Table 2 shows the rate constants of the first-order reactions for the luminescence activity at different NaCl gradients at pH 6.5 and 8.5. The constants at these pH values are seen to change depending on the ion concentration gradient. With a decrease in the NaCl gradient, the luminescence rate constant decreased in both acid and alkaline media. In the absence of the NaCl gradient, a 20-min incubation virtually did not change the pH-dependence profile of luminescence with its maximum at pH 7.5 (Fig. 2).

Table 2. Rate constants of luminescence activation (k_a, pH 6.5) and damping (k_d, pH 8.5) for cells loaded with 0.68 M NaCl at different NaCl concentrations in 25 mM Tris–HCl buffer

[NaCl] in the incubation medium, M	k_a, min^{-1} (pH 6.5)	k_d, min^{-1} (pH 8.5)
0.17	0.22	0.15
0.34	0.19	0.12
0.52	0.14	0.08

Figure 3 shows the pH dependencies of the luminescence intensity of cells loaded with 0.54 M KCl after 9, 15, and 20 min of incubation in medium with 0.4 M KCl. The course of the curves changed little with time; maximum luminescence was observed in a wide pH range from 6.5 to 7.5. The same pattern was observed with a decrease in the KCl concentration in the medium to 0.13 M; however, due to an extremely low level of luminescence at this KCl concentration (Table 1), it was impossible to reliably determine the pH-dependence profile.

The respiratory activity of intact cells was also controlled by Na^+ ions. Table 3 presents data on the ratios between the luminescent and respiratory activities (on the endogenous substrates) for cells loaded with 0.26 or 0.68 M NaCl and incubated in acid (pH 6.5) or alkaline (pH 8.5) medium. In cells loaded with 0.68 M NaCl, respiration and luminescence changed in synchronism at both pH 6.5 and 8.5 and did not depend on the concentration of extracellular NaCl: their ratio was virtually the same under all test conditions. A decrease in the total NaCl concentration (internal, 0.26 M; external 0.17 M) affected the respiratory activity to a greater extent than the luminescent activity. Under these conditions, the ratio of the luminescent activity to the oxygen consumption rate decreased 2- to 3-fold as compared to cells loaded with higher salt concentrations at either of the pH values.

Below, we present the results of the experiments on the action of CCP on the luminescent activity of bacteria in acid and alkaline media.

Cells loaded with 0.26 or 0.68 M NaCl were introduced in the incubation medium containing 0.17 or 0.52 M NaCl and 20 μM CCP at pH 6.5 or 8.5. The luminescence of the control samples (without CCP) was taken to be 100% for each system. The luminescent

activity of the cells was assessed after 5 min of incubation with the protonophore. CCP almost completely inhibited cell luminescence at pH 6.5 (residual activity, 1 to 2%) in all of the systems analyzed. At pH 8.5, the residual activity insignificantly depended on the extracellular NaCl concentration and was maintained at a level of about 50% for cells loaded with 0.26 M NaCl and 30% for cells loaded with 0.68 M NaCl.

DISCUSSION

Na^+ ions are known to be indispensable for the growth and luminescence of marine luminous bacteria. The physiological range of NaCl concentrations is wide (from 0.17 to 1.04 M) and is specific for each species [13]. The functional role of Na^+ in bioluminescence was suggested to be the creation of the required ionic strength and maintenance of the osmotic pressure [17].

A new concept of the role of Na^+ in luminescence was put forward by Wada *et al.* [10]: the supposition was advanced that luminous bacteria have an electrogenic Na^+ pump realized by the work of the NADH-dehydrogenase complex of the respiratory chain. Takuda and Unemoto [3–6] provided evidence of the generation of $\Delta\mu Na^+$ by *V. alginolyticus, V. costicola*, and other species of marine bacteria. In more than 10 species of marine bacteria of the genera *Vibrio, Alteromonas*, and *Alcaligenes*, the NADH–quinone oxidoreductase of the respiratory chain provides for the translocation of Na^+ from the cell into the alkaline medium, which generates on the plasma membrane the potential with a "+" sign outside [1]. The model of the coupling of electron transport and Na^+ translocation during NADH oxidation in *V. alginolyticus* is represented in [2, 5].

In this work, the involvement of the Na^+ pump in the transformation of chemical energy into light energy in photobacteria is considered. Prerequisites for such an approach are the membrane localization of the luminescent system [18], the specific structural features of the luminescent system composed of NADH dehydrogenase and luciferase, and the cells' requirement for NaCl for growth and luminescence.

One of the criteria of the operation of a Na^+ pump is the higher affinity of bioluminescence to Na^+ in comparison with other monovalent cations [10]. However, according to Wada *et al.* [10], this criterion is not fulfilled in acid media at pH 6.5. The data shown in this work, as distinct from [10], point to the high affinity of the luminescent reaction to Na^+ in the whole pH range studied (6.5 to 8.5). The bioluminescent activity primarily depends on the intracellular NaCl concentration and can be nonstationary in the 20-min range in case of a nonequilibrium distribution of sodium ions on the plasma membrane. These reasons can account for the discrepancy between the data on the efficiency of Na^+ and K^+ in acid media.

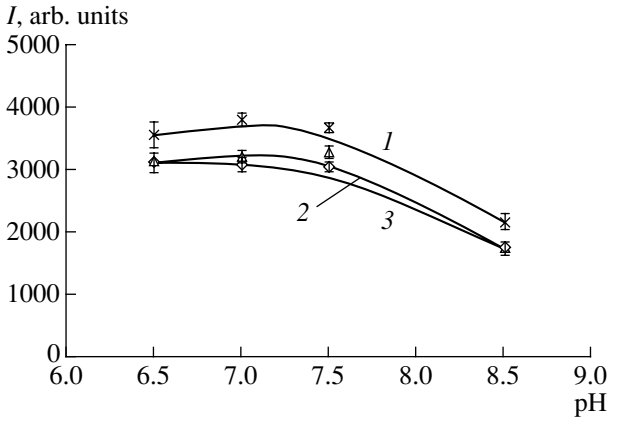

Fig. 3. pH dependence of the luminescence (*I*, arbitrary units) of cells loaded with 0.54 M KCl after (*1*) 9, (*2*) 15, and (*3*) 20 min of incubation in 25 mM Tris–HCl buffer containing 0.4 M KCl.

It is generally agreed that the optimum of the luminescent activity of all species of photobacteria in vivo is at pH 6.5–7.0. However, when *P. phosphoreum* was cultivated continuously, maximum luminescent activity was observed at pH 8.5 at 0.52 to 0.86 M NaCl in the cultivation medium [19].

The maximum level of the emission activity at pH 8.5 in 0.3 M of NaCl was noted for intact cells of *V. harveyi* [10]. The nonstationary emission observed in acid and alkaline media in the case of a nonequilibrium distribution of Na^+ ions may explain the contradictions between the cell luminescence pH optima determined by different authors and is in agreement with the results of [13], who conclude that salt concentration and pH produce a combined effect on the intensity and kinetics of light emission by photobacteria.

The fact that the rate constants of the bioluminescent reaction depend on the Na^+ ion concentration gradient supports the assumption that there exists a relationship between the Na^+ pump and the luminescence. Luminescence damping in alkaline media is likely to be determined by passive Na^+ diffusion along the concentration gradient, resulting in a decrease in the intracel-

Table 3. Ratios of luminescence (*Q*) of *V. harveyi* cells to their respiration (*V*) at different concentrations of NaCl in the medium at pH 6.5 and 8.5

pH	[NaCl] in the medium, M	Q/V, $\times 10^{11}$, quanta/nmol O_2	
		Cells loaded with 0.26 M NaCl	Cells loaded with 0.68 M NaCl
6.5	0.17	23	12
	0.52	8	13
8.5	0.17	19	11
	0.52	9	11

lular NaCl concentration and, consequently, in the membrane potential. An increase in the activity in acid media during incubation is rather difficult to explain. A decrease in the intracellular Na^+ ion concentration is probably compensated for by the nonequivalent transfer of H^+ ions into the cell, which causes the acidification of the intracellular medium and, as a consequence, activates a certain stage of the luminescent process, probably the formation of intermediate I ($E–FMNH_2$).

The stationary luminescence level was attained within 20 to 30 min. These results are consistent with the data obtained with another species of photobacteria *P. phosphoreum*. It was shown [14] that an increase in the NaCl concentration in the medium from 0.17 to 0.52 M activates luminescence; the transition to a new energy state occurs within 30 to 45 min. By direct analysis of ions using a plasma photometer, Nakamara *et al.* [20] showed that in medium with pH 8.9 and an ion concentration of 0.4 M, K^+ exit and Na^+ and Li^+ entrance in *V. alginolyticus* cells occur approximately within the same time (about 20 min) as the luminescence damping in *V. harveyi* that we observed at pH 8.5.

According to Wada *et al.* [10], the synchronism of changes in the respiration and luminescence caused by lithium, rubidium, potassium, and sodium ions is observed only in alkaline medium at pH 8.5. Our simultaneous analyses of the respiratory and luminescent activity at different pH and Na^+ ion gradients revealed synchronism of the changes in these processes in both acid and alkaline media in cells loaded with high NaCl concentrations. These experimental findings are explainable by taking into account the fact that the generation of $\Delta\mu Na^+$ is afforded by the work of NADH: FMN (quinone) oxidoreductase, which is the common segment of the respiratory and luminescent chains of electron transport.

The experiments on the identification of the Na^+ pump in *V. alginolyticus, V. costicola*, and other species of bacteria established that the uncoupler CCP efficiently inhibits their growth in acid media and exerts an insignificant effect in alkaline media, even when its concentration was increased to 10 to 50 μM [1, 2, 4]. The inefficiency of CCP in alkaline media was interpreted as direct evidence of the Na^+ pump operating under alkaline conditions. In this work, we studied the influence of CCP in cell suspensions at different concentrations of intracellular Na^+ and also revealed significantly less efficiency of CCP at pH 8.5 than at pH 6.5.

It should be noted that, at pH 8.5, the equilibrium shifts towards the anionic CCP form, which may also be the cause of less efficiency of its action under these conditions. The inhibitory effect of CCP on the luminescence of bacteria and bacterial luciferase is also known; however, as shown in Malkov's work [21], the sensitivity of *V. fischeri* cells to CCP at pH 7.0 is two orders higher than the sensitivity of luciferase. The inhibition of luciferase by this compound manifests itself at concentrations over 50 μM and is mediated via the hydrophobic mechanism. These data allow the possibility of the nonspecific action of CCP on the luminescence of bacterial cells to be excluded.

On the whole, the results obtained in this work (high affinity of luminescence to sodium ions, its resistance to high CCP concentrations in alkaline media, the dependence of the luminescence kinetics on the Na^+ ion gradient on the plasma membrane) agree with the data obtained with nonluminous species of marine bacteria [1–6] and *V. harveyi* [10] and testify in favor of the supposition of the generating of $\Delta\mu Na^+$ in photobacteria and its relation to luminescence.

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