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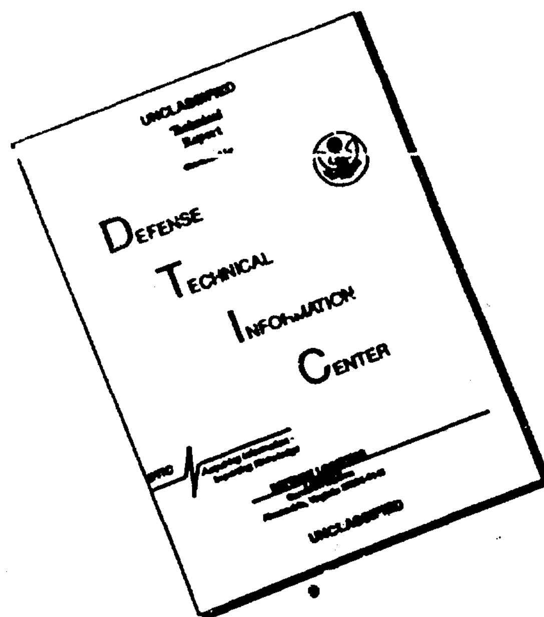

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The Connection of Bioluminescence of Bacteria with Respiration

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As is well known, bacterial bioluminescence is a chemoluminescence catalyzed by enzymes. The excited states of the luminescent molecules are created as the result of a particular chemical reaction: oxidation of a substrate luciferin in the presence of the enzyme luciferase. Bacterial luminescence is closely connected with processes of metabolism in the cell. This is evidenced by the following facts: 1) only living cells show luminescence; 2) the luminescence of intact bacteria occurs without any stimulation, continuously for a long time (under optimum conditions), whereas bacterial luminescence in vitro, which occurs when luciferase obtained from bacterial extracts is mixed with flavin mononucleotide, diphosphopyridine nucleotide and an aliphatic aldehyde, is quenched in several seconds. Despite the quite extensive literature on bacterial bioluminescence, the place and role of the light-emission reaction in the general cell metabolism have not yet been determined. To date, it remains unclear by which means the bacterial bioluminescence is included in metabolic processes.

In the work of a number of authors (1, 2) the opinion has been expressed that luminescent reactions are side-chains of certain normal metabolic processes, that is, that bioluminescence must take its origin from some slight changes in cell reactions common to all living organisms. Harvey considers the main oxidative chain -- respiration -- such a chain in cell metabolism which gives rise to bacterial bioluminescence, since it has been determined that bacteria luminesce only in the presence of oxygen.

In connection with what has been presented above, it was of interest to study the connection between bacterial bioluminescence and respiration. For the purpose of determining the degree of this relationship a study was made of the temperature relationships of both processes in the present work, since it is well known that the rate of processes catalyzed by enzymes depends a great deal on the temperature. For the purpose of clarifying the question of the intermediate area of the oxidative chain in which oxygen is used, which participates directly in the reaction of luminescence, we made a study of the effect of inhibitors of respiratory enzymes on luminescence and oxidation. As inhibitors KCN and thorazine were used. The inhibitory effect of KCN, as is well known, is based on the binding of the iron in the prosthetic group of cytochrome oxidase. According to the data of Yagi and co-worker (3), thorazine suppresses the activity of flavin enzymes. In

addition, there are data to the effect that thorazine quenches the excited states of molecules. Thus, Szent-Györgyi (4) reports that thorazine in the concentration of $2 \cdot 10^{-5}$M suppresses the phosphorescence of riboflavin and at a concentration of $2 \cdot 10^{-4}$M, the phosphorescence of acridine and rhodamine B. We have not encountered any work on the study of the effect of thorazine on bacterial bioluminescence.

Material and Method

The study was made with the following types of luminescent bacteria: Bacterium Issatschenkoi Egorova, Photobacterium sp. Kriss and with one of six strains of Photobacterium sp. luminescent bacteria isolated from the Pacific Ocean during the 34th voyage of the "Vityaz'." All the bacteria were kept in pure culture.

Determination of the Relationship of the Intensity of Bacterial Luminescence to the Temperature

For the purpose of measuring the intensity of the bacterial luminescence a special glass cuvette was designed which could be incubated and to which air was constantly supplied for mixing and aeration of the bacterial suspension. The temperature in the cuvette was measured with a thermocouple. The bacterial luminescence was recorded with a FEU-19 photomultiplier, the signal from which was fed after amplification to an EPP-09 automatic recording potentiometer. On the strip of the recorder a record was obtained of the intensity of luminescence. The luminescent bacteria were grown in test tubes on an agar slant. After the maximum brightness of luminescence was attained (from three to 24 hours for different species of bacteria) the bacteria were washed off the agar with 0.125 M phosphate buffer (pH = 7.1) containing three percent sodium chloride solution. The bacteria were washed off by centrifugation and were resuspended in a phosphate buffer with three percent NaCl solution and three percent glucose solution (the energy substrate). For the purpose of recording luminescence two cc of a luminescent suspension was put into the cuvette. At each temperature the record was made for five minutes.

Determination of the Relationship between the Rate of Oxygen Consumption (Respiration) and the Temperature

The respiratory rate of luminescent bacteria was determined by the manometric method in a Warburg apparatus. The vessels were calibrated with mercury by the Umbright and others' method (5). The brightly luminescent colonies of bacteria (three-five hours after streaking) were

washed off the agar with 0.125 M phosphate buffer containing three percent NaCl solution and were washed off by centrifugation. After removal of the centrifugate a suspension was prepared from the precipitate in the phosphate buffer containing three percent NaCl solution. The bacterial count per cc of the suspension was determined by counting in a Goryayev chamber /counting chamber/.

Three percent glucose solution was the energy substrate. One cc of the bacterial suspension and one cc of glucose solution were introduced into each of the Warburg vessels.

In the inner container 0.2 cc of 10 percent KOH was placed for absorption of the carbon dioxide which formed.

For each experiment two or three parallel manometers were used. At each temperature two-three measurements were made every 10 minutes (10 minutes after keeping the mixture at the given temperature). The oxygen consumption was expressed in microliters of oxygen per hour per cell (Q_{O_2}).

Study of the Effect of KCN and Thorazine on Growth, Luminescence and Respiration

Szent-Györgyi (4) points out that in a concentration of $1.3 \cdot 10^{-3}$M thorazine kills a mouse; from $7 \cdot 10^{-4}$ to $4.5 \cdot 10^{-5}$M it produces almost the same effect by reducing the metabolic rate in the mice by half; and at $2 \cdot 10^{-5}$M the effect of thorazine begins to decrease. We used the same thorazine concentrations. The potassium cyanide concentration in the experiment was equal to 10^{-3}M. First of all, we made a study of the effect of thorazine and KCN on the growth of luminescent bacteria. For this purpose the brightly luminescent colonies of Photobacterium sp. bacteria were washed off with nutri nt bouillon. From the washing obtained a suspension at a certain concentration was prepared (about 10^6 cells per cc). Thorazine was added to four small flasks containing the same quantity of bacterial suspension (25 cc) in a quantity such that the concentration of the inhibitor was equal to $1.3 \cdot 10^{-3}$; $7 \cdot 10^{-4}$; $4.5 \cdot 10^{-5}$; $2 \cdot 10^{-5}$M, respectively. The fifth small flask served as a control. All five flasks were placed in an incubator at 30° C. The growth of bacteria was determined by the density of the suspension measured on a photoelectric colorimeter-nephelometer, the FEKN-57. The effect of potassium cyanide on growth was judged by the survival of bacteria, which was determined by counting the colonies in Petri dishes.

For the purpose of studying the thorazine and KCN effect on luminescence and respiration Photobacterium sp. was used.

During the recording of luminescence of the bacterial suspension solutions of thorazine and KCN (10^{-2} and 10^{-3}M) were added to the

cuvette in quantities necessary for obtaining the corresponding concentrations of the inhibitor. The intensity of luminescence prior to the addition of the inhibitor was taken as 100; under the influence of thorazine and KCN it was expressed in percentages of the initial intensity of luminescence. During the experiment a temperature optimal for luminescence of the given species of luminescent bacteria was maintained in the cuvette. The suspension in the cuvette was continuously aerated.

In studying the effect of thorazine and KCN on respiration a certain quantity of inhibitor was put into the side retorts of the small vessels of the Warburg container the main volume of which was occupied by the suspension of luminescent bacteria. After keeping the vessels for 10 minutes at the temperature of the experiment the inhibitors were added to the bacterial suspension. The small vessel in which no inhibitor was added to the suspension was a control. Usually, there were two control vessels. The oxygen consumption was measured every 15 minutes for an hour. The number of bacteria per cc of suspension was determined by means of counting in a Goryayev chamber. The oxygen consumption (microliters of oxygen per hour per cell) in the control vessel was taken as 100; the oxygen consumption in the experimental vessels was expressed in percentages of the control.

Results and Discussion

Relationship of Luminescence and Respiration of the Bacteria to the Temperature

As for all biological processes, there is an optimum temperature for bacterial luminescence at which the intensity of luminescence is at a maximum. At temperatures lying below and above the optimum temperature, the intensity of luminescence decreases. The curves which we obtained of the "intensity of luminescence versus the temperature" for the species of luminescent bacteria studied are shown in Fig. 1. From the Figure it is seen that Bacterium Issatschenkoi Egorova, Photobacterium sp. Kriss and Photobacterium sp. show different optimum temperatures for luminescence. A study of the respiration of Bacterium Issatschenkoi Egorova, Photobacterium sp. Kriss and Photobacterium sp. showed that the relationships between the oxygen consumption by luminescent bacteria and the temperature is the same for all three species. When the temperature was increased to 36-38° C the oxygen consumption by the bacteria increased; further increase in the temperature led to a reduction of oxygen consumption. The maximum respiratory rate of Bacterium Issatschenkoi Egorova and Photobacterium sp. Kriss is observed at 36-37°; of Photobacterium sp., at 38° (Fig. 2).

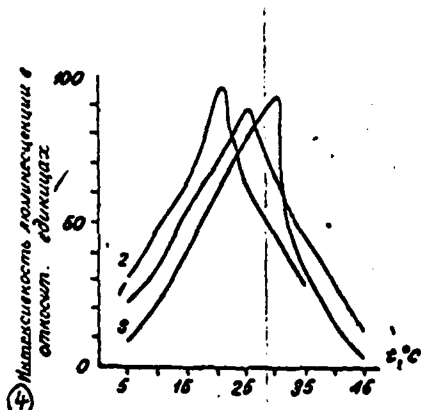

Fig. 1. Relationship Between the Intensity of Bacterial Luminescence and the Temperature. 1. Bacterium Issatschenkoi Egorova; 2. Photobacterium sp. Kriss; 3. Photobacterium sp.; 4. intensity of luminescence in relative units.

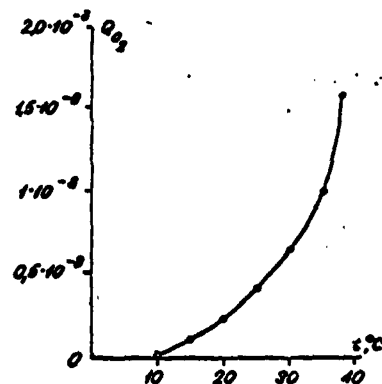

Fig. 2. Relationship Between the Respiratory Rate of Photobacterium sp. and the Temperature.

The studies showed that the temperature relationships of luminescence are of an entirely different character from the temperature relationships of respiration. For each species of luminescent bacteria the optimum temperatures of luminescence and respiration are different. Thereby, the temperature of greatest oxygen consumption is always higher than the temperature of maximum intensity of luminescence. Thus, at the optimum temperature for luminescence of Photobacterium sp. (30°) the oxygen consumption is 2.5 times less than at 38° (Fig. 2).

Therefore, no proportional relationship was found between oxygen consumption and the rate of the reaction of luminescent oxidation of luciferin as determined by intensity of luminescence. Study of the temperature relationships of luminescence and oxygen consumption in Bacterium Issatschenkoi Egorova, Photobacterium sp. Kriss and

Photobacterium sp. showed that with the same temperature relationships of respiration in all three species of bacteria different relationships of luminescence to the temperature are observed. For each species of luminescent bacteria studied there is a characteristic curve of "intensity of luminescence versus the temperature," which in its nature and principally in the location of the maximum is different from similar curves for other species. Therefore, the data obtained in the present work are evidence to the effect that luminescent reactions of bacteria, inseparably connected with the chain of oxidation, are specific and different from those reactions by means of which the stepwise transfer of electrons is accomplished from the substance being oxidized to oxygen. However, the study of the temperature relationships of luminescence and respiration did not lead to a solution of the problem of how the relationship between the light-emitting reaction and oxidative processes is realized. We proposed to obtain the data on the segment of the respiratory chain at which oxygen enters the luminescent reaction by using simultaneous inhibition of luminescence and respiration.

The Effect of Thorazine and Potassium Cyanide on the Growth, Luminescence and Respiration of Luminescent Bacteria

It was shown in the present work that thorazine in concentrations from $1.3 \cdot 10^{-3}$ to $2.0 \cdot 10^{-5}$M exerts different effects on the activities of luminescent bacteria. The addition of thorazine in a concentration of $1.3 \cdot 10^{-3}$M to the bacterial suspension immediately leads to cell lysis. Thorazine in the concentration of $7 \cdot 10^{-4}$M first stops the division of bacteria and after 10-15 minutes kills them (the results of the determination of bacterial survival by the method of counting colonies on Petri dishes).

The effect of thorazine in a concentration of $4.5 \cdot 10^{-5}$M reduces the rate of bacterial growth, as the result of which four hours after streaking the density of the suspension being incubated with thorazine is less than the density of the control suspension by 26.8 percent. Thorazine in a concentration of $2 \cdot 10^{-5}$M exerts a very slight effect on bacterial growth. After four hours, the density of the suspension being incubated with this concentration of thorazine is only 12 percent less than the density of the control suspension. The determination showed that the effect of potassium cyanide in a concentration of 10^{-3}M has no influence on the activity of luminescent bacteria.

Study of the effect of KCN in a concentration of 10^{-3}M on the luminescence and consumption of oxygen showed that potassium cyanide is an inhibitor of respiration only. In a concentration of 10^{-3}M it reduces the oxygen consumption by 85-90 percent. These data attest to the fact that

cytochrom apparently does not participate in bacterial luminescence. Oxygen does not enter the light-emitting reaction at the end of the oxidative chain but before that, at some intermediate segment of it.

It was found that thorazine is an inhibitor of both the luminescent system and of respiration. Data on the effect of different thorazine concentrations on the intensity of luminescence and oxygen consumption are given in the Table.

The Effect of Thorazine on the Intensity of Luminescence and Respiration of Photobacterium sp.

(1) Концентрация аминазина, М	(2) Подавление интенсивности люминесценции, %	(3) Подавление потребления кислорода, %
$7 \cdot 10^{-4}$	100	100
$4,5 \cdot 10^{-5}$	30	35
$2 \cdot 10^{-6}$	20	26
$1 \cdot 10^{-5}$	Не влияет (4)	20

Note. The intensity of luminescence and oxygen consumption were determined at 30° C.
1. thorazine concentration, M; 2. quenching of the intensity of luminescence, percent; 3. inhibition of oxygen consumption, percent; 4. has no influence.

Simultaneous inhibition of luminescence and respiration by thorazine attest to the fact that the inhibitor acts on some components of the chain of oxidation common to luminescence and respiration. It is our suggestion that this component in the chain of oxidation in bacteria is represented by flavin enzymes. Through the work of Strehler (6), McElroy and others (7) it has been found that the luminescence of bacterial extracts depends on the quantity of flavin mononucleotide added. However, no proof has been obtained of the direct participation of flavin enzymes in the bioluminescence of intact bacteria. In our opinion, the present work is evidence to the effect that flavins participate directly in bacterial bioluminescence.

Conclusions

1. Luminescence and respiration in luminescent bacteria have different optimum temperatures. The optimum for oxygen consumption is at a higher temperature than the optimum for luminescence;

2. Luminescent reactions of bacteria, closely connected with

metabolic processes, are specific reactions. Luminescence and respiration are two different processes.

3. Apparently, cytochromes do not participate in bacterial luminescence, and

4. The connection between the luminescent system of bacteria and respiration is realized through flavin enzymes.

In conclusion we should like to express our deep appreciation to Professor I. I. Gitel'zon and Professor I. A. Terskov for their guidance in this work; to I. K. Damova for assistance in performing the experiments.

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