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Effect of tritium on luminous marine bacteria and enzyme reactions

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ABSTRACT

The paper studies chronic effect of tritiated water, HTO, (0.0002–200 MBq/L) on bioluminescent assay systems: marine bacteria *Photobacterium phosphoreum* (intact and lyophilized) and coupled enzyme reactions. Bioluminescence intensity serves as a marker of physiological activity. Linear dependencies of bioluminescent intensity on exposure time or radioactivity were not revealed. Three successive stages in bacterial bioluminescence response to HTO were found: (1) absence of the effect, (2) activation, and (3) inhibition. They were interpreted in terms of reaction of organisms to stress-factor i.e. stress recognition, adaptive response/syndrome, and suppression of physiological function. In enzyme system, in contrast, the kinetic stages mentioned above were not revealed, but the dependence of bioluminescence intensity on HTO specific radioactivity was found. Damage of bacteria cells in HTO (100 MBq/L) was visualized by electron microscopy. Time of bioluminescence inhibition is suggested as a parameter to evaluate the bacterial sensitivity to ionizing radiation.

1. Introduction

Tritium, beta-emitting radionuclide, is permanently generated by space radiation at the rate of 1200 atoms s^{-1} m^{-2} in the top layers of earth atmosphere (Lenskii, 1981). In environment, it is mostly presented as a component of tritiated water (HTO). Until 50-ties the tritium concentration in natural waters was low – one tritium per 10^{18} hydrogen atoms. However after atmosphere nuclear tests it increased 1000-fold. Since a half-life of tritium is 12.32 years, its concentration eventually decreased, though local rise of tritium content took place around nuclear power plants. Local nuclear incidents increase tritium concentration dramatically. In future, controlled fusion reactors can bring an additional threat of contamination with tritium.

Tritium is considered to be one of the less dangerous isotopes. Maximal energy of its beta-particles is low (18.59 keV) and maximal range of their path is short (5.8 mm at 20 °C). Beta-particles are entirely absorbed by skin's surface layers; this is why tritium is not dangerous outside an organism. However, as an internal source of irradiation it can be unsafe. Tritium can substitute hydrogen atoms in organic molecules and is capable of penetrating into cell organelles. Free electrons and helium-3 cations are products of tritium radioactive decay. Helium-3 cation is a very active particle; it can withdraw electron from organic molecules forming stable electron shell of inert gas, helium, and active carbenic cation. This is why the tritium irradiation can produce a local damage. Tritium specific ionization ability (2.2×10^6 ions per cm) exceeds that of other beta-emitting radionuclides. Hence, tritium toxicity is a challenging problem for researchers working in related fields (Evans, 1974; Snigireva et al., 2009).

Recent years have seen a change in the approach in radiobiological studies: biota *in toto* might be considered as a target of radiation impact, with the human included as a part of biota and integrated into biosphere by multiple functional interrelations. Microorganisms are the simplest and basic part of biosphere, and their physiological indices serve as indicators of biosphere state on the whole. Hence, microorganisms can be used as bioassays to monitor environmental radiotoxicity.

As a matter of fact, the main feature of all bioassays is an integral response that accounts for non-additivity of the effects of numerous environmental pollutants and natural components. It implies that toxic effect of a sum of compounds can be higher or lower than the sum of the effects of these compounds. Radiometric and chemical analyses *per se* do not evaluate hazard to living organisms;

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they take no account of non-additivity of the effects and differences in sensitivity of various organisms. Only a set of radiometric, chemical, and biological methods can provide with complete information on ecological state of a medium (Kudryasheva et al., 1998). It was supposed that this set should involve bioassays of different sensitivity to toxic compounds.

Assay systems based on luminous marine bacteria are good candidates for radiotoxicity monitoring. Bacterial bioluminescent assays have been widely used to monitor environmental toxicity for more than forty years (Kuts and Ismailov, 2009; Girotti et al., 2008; Ivask et al., 2009; Kudryasheva et al., 2003; Roda et al., 2009; Thomas et al., 2009), and now they are conventional and important biotechnological applications of bioluminescence phenomenon. The tested parameter here is luminescence intensity that can be easily measured instrumentally. The advantages of bioluminescent assays are their high sensitivity, simplicity and rapidity of measurements (1–3 min), and availability of simple devices to register toxicity (Gitelson and Kratasyuk, 2002).

The bacterial bioluminescent assays can involve biological systems of different organization – bacteria or enzymes. Bacterial bioluminescent enzyme system was suggested as a bioassay for the first time in 1990 (Kratasyuk, 1990). Advantages for this approach were demonstrated later (Roda et al., 2009; Girotti et al., 2008; Esimbekova et al., 2007; Kudryasheva et al., 2002). Primary physico-chemical processes in bioluminescent enzyme system were reviewed (Nemtseva and Kudryasheva, 2007); mechanisms of exogenous compounds interactions with enzyme systems were discussed (Kudryasheva, 2006).

For the first time, the bacterial bioluminescence was used to monitor radiation toxicity in (Min et al., 2003). In this work, the effect of gamma-ray irradiation on recombinant *E. coli* strain was studied. Effects of ionizing radiation, in the form of a proton beam from a cyclotron, on kinetics of firefly luciferase reaction were revealed (Berovic et al., 2008), they were explained by elimination of dissolved oxygen in aqueous solutions. In our previous works (Rozhko et al., 2007, 2008, 2011; Alexandrova et al., 2011), we studied sensitivity of bioluminescent assay systems to Americium-241, alpha-emitting radionuclide, product of plutonium radioactive decay. The effect of tritium-labeled amino acid valine (0.3–1.0 MBq/mL) on luminous bacteria *Photobacterium phosphoreum* was studied in (Alexandrova et al., 2010); the tritium-labeled valine was used as a component of nutrient medium for the bacteria. It was found to suppress bacterial growth, but stimulate luminescence: luminescence intensity, quantum yield, and time of light emitting increased.

A number of researchers (Kuzin, 1994; Nagasawa and Little, 1999; Nikolsky and Koterov, 1999) assumed that there is a relationship between the level of organization of a biological object and its response to radiation exposure, and this can be of both theoretical and practical interest. Bioluminescent assay systems can be conveniently used to verify this assumption as they can be based on biological objects of different levels of organization – bacteria-based or enzyme-based bioassays; these bioassays inform about toxic effects on cells or enzymes, respectively (Girotti et al., 2008; Fedorova et al., 2007). In (Rozhko et al., 2007) the effect of Americium-241 was evaluated in these two bioassays, and bacterial assay showed higher sensitivity.

The purpose of the work was to investigate the effect of tritium (as tritiated water, HTO) on bioluminescent assay systems of different complexity (bacteria-based and enzyme-based assays) under conditions of chronic irradiation. We aimed at bioluminescent intensity, bacterial growth, cell damage, and tritium accumulation.

The applied aspect of our work focuses on elaboration of scientific basis for monitoring toxicity of tritiated water using bioluminescent assays. We consider the bioluminescent intensity of assay systems at various time of exposure to HTO and different HTO radioactivities. Responses of the bioluminescent assays are compared. Use of bacterial assays of different type (intact or lyophilized bacteria) provides comparison of their sensitivity to ionizing radiation.

2. Materials and methods

Three bioluminescent assays were applied:

assay 1 intact bacteria, i.e. cell suspension of 20-h culture *Photobacterium phosphoreum* 1883 IBSO from the Collection of Luminous Bacteria IBSO-836;

assay 2 Microbiotest 677F preparation of lyophilized *P. phosphoreum*1883 IBSO (Kuznetsov et al., 1996);

assay 3 enzymatic system, i.e. a preparation based on a coupled enzyme system: NADH:FMN-oxidoreductase from *Vibrio fischeri* (0.15 a.u.) and luciferase from *Photobacterium leiognathi*, 0.5 mg/mL (Gitelson and Kratasyuk, 2002; Kuznetsov et al., 1996).

All the biological preparations were produced at the Institute of Biophysics SB RAS, Krasnoyarsk, Russia.

The chemicals used were: NADH from ICN, USA; flavinmononucleotide (FMN) and tetradecanal from SERVA, Germany.

HTO (radiochemical purity 98%) of specific radioactivities 0.0002, 0.002, 0.02, 0.2, 2, 10, 20, 50, 100, and 200 MBq/L was applied as a source of ionizing radiation.

Bioluminescent intensities of control (without HTO) and tested (with HTO) samples were examined and compared in assays 1–3.

Intact bacteria (assay 1) were grown in 30 mL nutrient media. To cultivate bacteria, the semisynthetic medium containing: 1 g/L KH_2PO_4, 6 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.5 g/L $(NH_4)_2HPO_4$, 0.2 g/L $MgSO_4$ $7H_2O$, 30 g/L NaCl, 5 g/L peptone, and 3 mL glycerol was used. Bacteria were grown at different HTO activities at pH 6.9–7.0. Optical density of bacterial suspensions (D) was registered using a KFK-2MP colorimeter, Russia.

Relative optical density D^{rel} was calculated as follows:

$$D^{rel} = \frac{D_{rad}}{D_{contr}} \quad (1)$$

where D_{rad} and D_{contr} are maximal optical densities in radioactive and control samples, respectively. Values of D^{rel} were plotted vs. time or tritium specific radioactivity.

The 100 μL bacterial suspension was sampled at the stationary stage of growth (20 h) for bioluminescent measurements (assay 1). The samples were kept under 4 °C, and incubated for 5 min at room temperature (20 °C) before the measurements.

The influence of HTO on bioluminescent intensity of lyophilized bacteria (assay 2) was studied in 0.3 μL bacterial solution in 1.5% NaCl and 150 μL HTO of different activity.

The conditions of bioluminescent measurements excluded growth of bacteria in assays 1 and 2.

To construct the enzyme system (assay 3), 0.1 mg/mL enzyme preparation, $5 \cdot 10^{-4}$ M FMN, $4 \cdot 10^{-4}$ M NADH, and 0.0025% tetradecanal solutions were used. The assay was performed in a 0.05 M phosphate buffer (pH 6.8) at room temperature. Test sample included: 2.5 μL enzyme preparation, 2.5 μL FMN, 2.5 μL tetradecanal, 100 μL NADH, and 100 μL HTO of different radioactivity. Bioluminescent reaction was initiated by NADH solution.

Bioluminescent intensity was registered by TriStar Multimode Microplate Reader LB 941, Berthold Technologies.

Relative bioluminescent intensity I^{rel} was calculated in all three assays according to: