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# Metabolism and Physiology of Halobacteria

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**Abstract**

Halophiles (*lat*. "salt-loving") is the taxonomic group of extreme aerobic microorganisms that live in conditions of high salinity – in the seas, salt lakes, saline soils etc. These microorganisms are known to reddish patina on products, preserved with using large quantities of salt (NaCl). Halophiles were isolated for the first time at the beginning of the XX century from the marine flora estuary mud, but their systematic study was started only at the end of the second decade of the XX century. The internal environment of the human body is not suitable for existence of halobacteria, since none of them are known to have pathogenic forms. Halobacteria have great practical potential for using in molecular bioelectronics and bio-nanotechnology due to their unique ability to convert the energy of sunlight into electrochemical energy of protons $H^+$ due to the presence in their cells a special photo transforming retinal containing integral protein – bacteriorhodopsin, the mechanism of action of which has been currently studied in detail. This article describes the characteristics of the metabolism and physiology of halophilic bacteria, as well as a method of biosynthesis and preparation of bacteriorhodopsin from purple membranes of cells of the extreme photoorganotrophic halobacterium *Halobacterium halobium*.
**Key words:** halobacteria, bacteriorhodopsin, purple membranes, biosynthesis.

## Introduction

The halophiles, related according to the taxonomic classification to the ancient archaea *Archebacteria* genera are single-celled microorganisms with no marked nucleus and expressed membrane organelles, occupy a special place among other microorganisms (Oren, 1993). These are the only microorganisms that can exist in environments with a high salt content – on salt crystals in the coastal strip, on the salt marsh, in the salt brine etc. (Figure 1). In the Dead Sea (Israel), for example, the salt concentration reaches 26–27 %, in some years, rising to 30 %, whereas at 35 % NaCl precipitates from the salt solution into the sediment (Shammohammadi, 1998). Biochemical apparatus of the cell, enzymes and ribosomes of halophilic bacteria due to the peculiar cell osmoregulation system and the structure of the cell wall, consisting of proteins and amino sugars, is not only insensitive to such high salt concentrations, but on the contrary needs NaCl and functions effectively only in saturated solutions with 15–20 % of NaCl (Lake et al., 1985). For maintainance of cell stability of halophiles primarily NaCl is required. Wherein, $Na^+$ cations interact with the negatively charged cell wall of halobacteria imparting the necessary stiffness. Inside the cell, the concentration of NaCl is low. Potassium cations ($K^+$), in conjunction with chlorine anions ($Cl^-$) are needed to maintain the ionic equilibrium inside and outside of the cells, to stabilize enzymes and other cellular membrane structures of halobacteria. While removing the halobacteria from salinity environment, their cell wall is dissolved and the cytoplasmic membrane disintegrates into smaller fragments.

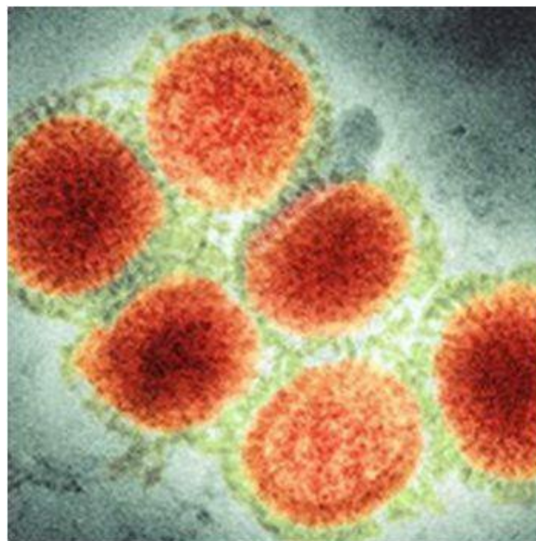

*Figure 1*: Red colored colonies of halobacteria in the salt marsh of the Dead Sea (Israel)

The family of halobacteria (lat. *Halobacteriaceae)* includes about 20 genera, including *Halobacterium*, *Halococcus*, *Haloarcula*, *Natrococcus*, *Natrobacterium* and others. Extremely halophilic forms of halobacteria are referred to the genera of *Halobacterium* and *Halococcus* (15–32 % NaCl), whereas less halophilic forms – to the genera of *Haloarcula*, *Natronobacterium* and *Natronococcus* (5–20 % NaCl). Members of the halobacteria family are presented by coccoid or rod-shaped forms, as well as by mobile or stationary forms of microorganisms, most of which are painted Gram-positively. Some halobacteria have gas vacuoles for keeping the buoyancy control. The halobacteria usually do hot form spores. According to the nutrition type halobacteria are predominantly aerobic microorganisms, e.g. they require oxygen for the growth, but they also can tolerate the very low oxygen content in growth media (hemoorganotrophs) (Ignatov & Mosin, 2014). Furthermore, halobacteria can use a wide range of organic compounds for the growth as amino acids, carbohydrates and organic acids. They possess a few changes in biochemical pathways of assimilation of sugars via complete citric acid cycle. With a lack of oxygen halobacteria are able to evolve the photoorganotrophic pathway by synthesizing the photo-transforming membrane protein bacteriorhodopsin (BR), which allows use solar energy for the growth.

The halobacteria are oftnen refered as organisms “living on the edge of physiological capabilities”. They have virtually no competitors that could exist under the same conditions, and therefore halophiles freely evolved throughout the evolution of life on Earth. The interesting fact is that some modern bacteria under the growth in extreme conditions acquire the features of halophiles and other ancient archaebacteria by losing the more rigid upper layer in the cell membrane. It is assumed that archaebacteria had lost this layer under the influence of high salt concentrations. The changes in the structure of their cell membranes are caused by the need to ensure the necessary protection for the cells from aggressive external environment. The process of formation of adaptive protective systems in halobacteria demanded the synthesis of specific substances and cellular systems, which do never almost occur in other microorganisms.

Halobacteria have lived on Earth since the Archean age – 3.0–3.5 billion years, almost without changing. Fossilized remains of these organisms are found in ancient rocks aged 2.7 billion years and in Precambrian formations. The oldest of these bacterial remains were found in Isuan green-stone belt in the west of Greenland, where there have been the oldest on Earth, sedimentary rocks formed 3.8 billion years ago. It is possible that the archaebacteria were the first forms of life on Earth in the first period of its evolution (Mosin & Ignatov, 2013). One of the main arguments in favor of this hypothesis is the fact that the representatives of numerous species of archaebacteria use as the sole carbon source for the biosynthesis of components of the cell biomass a mixture of amino acids, i.e., they are heterotrophs.

It is argued that the archaea, bacteria and eukaryotes are submitted by three separate taxonomic lines which were early separated from the ancestral group of microorganisms (Pfeiffer et al., 2008). Perhaps it was occurred even before the evolution of the cell, wherein the lack of the cell membrane made it impossible to unlimited transfer and exchange of genes that is why the ancestors of the three domains are differed in lockable sets of genes. It is very likely that the common ancestor of archaea and bacteria was a thermophile, it gives a reason to us to assume that low temperatures were “extreme environment” for archaea, and organisms adapted to them, appeared a little later. Indicating the relationship between these three domains is a crucial for understanding the origin of life. The majority of metabolic pathways, which involves most of the genes are similar in bacteria and archaea, whereas the genes responsible for the expression of other genes are very similar in archaea and eukaryotes. According to the cellular

structure the archaea are closest to Gram-positive bacteria: their cells are covered with the plasma membrane, the additional outer membrane, characteristic for Gram-negative bacteria is absent; the cell walls are of varying composition and as a rule are usually thick.
Table 1 below shows some of the main features of the archaea, characteristic or inherent to other domains, to demonstrate their relationship.

*Table* 1: The general features characteristic of bacteria, archaea and eukaryotes

| Typical for archaea and bacteria | Typical for archaea and eukaryotes | Typical only for archaea |
|---|---|---|
| The absence of the nucleus and membrane organelles | The absence of peptidoglycan (murein) | The structure of the cell wall (cell walls of some archaea contain psevdomurein) |
| Ring chromosome | DNA is associated with histone protein | The cell membrane lipids contain ether linkage bond |
| Genes are combined into operons | Translation of protein begins with the methionine residue | The structure of flagellin |
| No introns and RNA processing | Similar RNA polymerase and other components of the transcription | The structure of the ribosome (some signs closer to the bacteria, while some others - with eukaryotes) |
| Polycistronic mRNA | Similar mechanisms of replication and repair of DNA | The structure and metabolism of tRNA |
| Cell size (more than 100 times less than in eukaryotes) | A similar ATPase (type V) | No fatty acid synthase |

The genetic apparatus of the archaea is represented by a single circular chromosome with the size of 5751492 bp found in *Methanosarcina acetivorans*, having the largest known gene among the archaea (Joshia, 1963). On the contrary in *Nanoarchaeum equitans* 1/10 the size of the genome composes 490 885 bp, having the smallest known genome among the archaea; it contains only 537 genes, encoding different proteins (Peck et al., 2000). The archaea also contains smaller molecules of DNA, known as plasmids. Probably the plasmids can be transferred via contact between the cells, in a process similar to bacterial conjugation.
However, evolutionary relationship between archaea and eukaryotes remains to be unclear. Besides the similarities in the structure and functions of cells, there are similarities at the genetic level. It was found that a group of archaea – *Crenarchaeota* are closer to eukaryotes than to other types of archaea – *Euryarchaeota* (Park et al., 2009). The most common is a hypothesis that the ancestor of eukaryotes early separated from the archaea, and eukaryotes appeared as a result of the merger of archaea and eubacteria; the later became the cytoplasm and the nucleus of the new merged cell (Woese, 1998). This hypothesis explains the various genetic similarities, but has some difficulty in explaining the cell structure.
The recent information on the genetic diversity of the archaea is fragmentary, and the total number of species could not be evaluated fully. Comparative analysis of the 16S structures of rPHK of archaea allowed assuming the existence of 18–20 phylogenetic groups of the archaea (Lake, 1988). Numerous of these groups are known only from a single sequence of rRNA, which suggests that the limits of the diversity of these organisms remain to be unclear. Many halobacteria have never been cultured under laboratory conditions, which makes their identification difficult.
The aim of the research was the investiging of the metabolism and physiology of extreme halophilic bacteria *Halobacterium halobium* and searching for new biotechnological applications of the synthesized by this bacterium photo-transforming integral membrane protein bacteriorhodopsin (BR).

 

## Material and Methods

***Bacterial objects***

As a BR producer was used a carotenoid strain of extreme photo-organotrophic halobacterium *Halobacterium halobium* ET 1001, obtained from Moscow State University (Russia). The strain was modified by selection of individual colonies on solid (2% (w/v) agarose) media with peptone and 4.3 M NaCl.

***Growth conditions***

BR (yield, 8–10 mg from 1 g biomass) was obtained in synthetic (SM) medium (g/l): *D,L*-alanine – 0.43; *L*-arginine – 0.4; *D,L*-aspartic acid – 0.45; *L*-cysteine – 0.05; *L*-glutamic acid – 1.3; *L*-lycine – 0.06; *D,L*-histidine – 0.3; *D,L*-isoleucine – 0.44; *L*-leucine – 0.8; *L*-lysine – 0.85; *D,L*-methionine – 0.37; *D,L*-phenylalanine – 0.26; *L*-proline – 0.05; *D,L*-serine – 0.61; *D,L*-threonine – 0.5; *L*-tyrosine – 0.2; *D,L*-tryptophan – 0.5; *D,L*-valine – 1.0; AMP – 0.1; UMP – 0.1; NaCl – 250; $MgSO_4 \cdot 7H_2O$ – 20; KCl – 2; $NH_4Cl$ – 0.5; $KNO_3$ – 0.1; $KH_2PO_4$ – 0.05; $K_2HPO_4$ – 0.05; $Na^+$-citrate – 0.5; $MnSO_4 \cdot 2H_2O$ – $3 \cdot 10^{-4}$; $CaCl_2 \cdot 6H_2O$ – 0,065; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $FeSO_4 \cdot 7H_2O$ – $5 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$; glycerol – 1.0; biotin – $1 \cdot 10^{-4}$; folic acid – $1.5 \cdot 10^{-4}$; vitamin $B_{12}$ – $2 \cdot 10^{-5}$. The growth medium was autoclaved for 30 min at 0.5 atm, the pH value was adjusted to 6.5–6.7 with 0.5 *M* KOH. Bacterial growth was performed in 500 ml Erlenmeyer flasks (volume of the reaction mixture 100 ml) for 4–5 days at 35 $^0$C on Biorad shaker ("Birad Labs", Hungary) under intense aeration and monochromatic illumination (3 lamps × 1.5 *lx*). All further manipulations for BR isolation were carried out with the use of a photomask lamp equipped with an orange light filter.

***Isolation of purple membranes (PM)***

Biomass (1 g) was washed with distilled water and pelleted by centrifugation on T-24 centrifuge ("Carl Zeiss", Germany) (1500 g, 20 min). The precipitate was suspended in 100 ml of dist. $H_2O$ and kept for 3 h at 4 $^0$C. The reaction mixture was centrifuged (1500 g, 15 min), the pellet was resuspended in 20 ml dist. $H_2O$ and disintegrated by infrasound sonication (22 kHz, 3 times × 5 min) in an ice bath (0 $^0$C). The cell homogenate after washing with dist. $H_2O$ was resuspended in 10 ml of buffer containing 125 mM NaCl, 20 mM $MgCl_2$, and 4 mM Tris-HCl (pH = 8.0), then 5 mg of RNA-ase (2–3 units of activity) was added . The mixture was incubated for 2 h at 37 ℃. Then 10 ml of the same buffer was added and kept for 10–12 h at 4 $^0$C. The aqueous fraction was separated by centrifugation (1500 g, 20 min), the PM precipitate was treated with 50 % (v/v) ethanol (5 times × 7 ml) at 4 $^0$C followed by separation of the solvent. This procedure was repeated 6 times to give colorless washings. The protein content in the samples was determined spectrophotometrically on DU-6 spectrophotometer ("Beckman Coulter", USA) by the ratio $D_{280}/D_{568}$ ($\varepsilon_{280} = 1.1 \cdot 10^5$; $\varepsilon_{568} = 6.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) (Mosin & Ignatov, 2013b). PM regeneration is performed as described in the article (Mosin et al., 2013). Yield of PM fraction – 120 mg (80–85 %).

***Isolation of BR***

Fraction PM (in $H_2O$) (1 mg/ml) was dissolved in 1 ml of 0.5 % (w/v) sodium dodecyl sulfate (SDS-Na), and incubated for 5–7 h at 37 $^0$C followed by centrifugation (1200 g, 15 min). The precipitate was separated, than methanol was added to the supernatant in divided portions (3 times × 100 ml) at 0 $^0$C. The reaction mixture was kept for 14–15 h in ice bath at 4 $^0$C and then centrifuged (1200 g, 15 min). Fractionation procedure was performed three times, reducing the concentration of 0.5 % SDS-Na to 0,2 and 0,1 %. Crystal protein (output 8–10 mg) was washed with cold $^2H_2O$ (2 times × 1 ml) and centrifuged (1200 g, 15 min).

***Purification of BR***

Protein sample (5 mg) was dissolved in 100 ml of buffer solution and placed on a column (150×10 mm), stationary phase – Sephadex G-200 ("Pharmasia", USA) (specific volume packed beads – 30–40 units per 1 g dry of Sephadex) equilibrated with buffer containing 0.1 % (w/v) SDS-Na and 2.5 mM ETDA. Elution proceeded by 0.09 M Tris-buffer containing 0.5 M NaCl, pH = 8.35 at a flow rate of 10 $ml/cm^2 \cdot h$. Combined protein fraction was subjected to freeze-drying, in sealed glass ampoules (10×50 mm) and stored in frost camera at -10 $^0$C.

***Quantitative analysis of the protein***

The procedure was performed in 12.5 % (w/v) polyacrylamide gel (PAAG) containing 0.1 % (w/v) SDS-Na. The samples were prepared for electrophoresis by standard procedures (LKB protocol, Sweden). Electrophoretic gel

 

stained with Coomassie blue R-250 was scanned on a CDS-200 laser densitometer (Beckman, USA) for quantitative analysis of the protein.

***Absorption spectra***
Absorption spectra of pigments were recorded on the programmed DU-6 spectrophotometer ("Beckman Coulter", USA) at $\lambda = 280$ nm and $\lambda = 750$ nm.

***Scanning electron microscopy***
The structural studies were carried out with using scanning electrom microscopy (SEM) on JSM 35 CF (JEOL Ltd., Corea) device, equiped with X-ray microanalyzer "Tracor Northern TN", SE detector, thermomolecular pump, and tungsten electron gun (Harpin type W filament, DC heating); working pressure: $10^{-4}$ Pa ($10^{-6}$ Torr); magnification: 300000, resolution: 3.0 nm, accelerating voltage: 1–30 kV; sample size: 60–130 mm.

**Results and Discussion**

***The structure of BR***

The mechanism of converting the solar energy into the chemical energy of ATP used by halobacteria is different from the classic photosynthetic mechanism realized by plants and green algae containing chlorophyll. For this purpose halobacteria use a special chromophore protein with a molecular weight of 26 kDa, designated bacteriorhodopsin (BR) by analogy with the photo-sensitive protein of mammalian visual apparatus – rhodopsin providing visual perception in animals and humans (Oesterhelt & Stoeckenius, 1971). BR was firstly isolated in 1971 from the cell membrane of extreme photoorganotrophic halobacteria *Halobacterium halobium*, inhabiting saline geothermal lakes and seas, including the Dead Sea (Israel) (Oesterhelt, 1976). This photo-transforming protein is represented by a chromoprotein associated by aldimine bond with the amino acid residue lysine-216. As a chromophore group the BR contains an equimolar mixture of 13-*cis*- and 13-*trans*-retinal – an analogue of vitamin A defining purple-red color of colonies of halobacteria. Along with the BR the cell membrane of halophiles contains other carotenoid pigments, the main of which bakterioruberin, causes the colorage of halobacteria from pink to red and red-orange (Ignatov & Mosin, 2013). The presence of these pigments has to the halophiles an important meaning as a means of protection against excessive solar radiation, as their habitats are characterized by high luminosity, and these pigments are able to delay radiation.
The cell membrane of halophiles also contains two sensory rhodopsins, which provide positive and negative phototaxis in cells (Mosin & Ignatov, 2014). These proteins absorb different wavelengths of light, causing a cascade of signals that eventually control the flagella of halobacteria. For example, the absorption of a photon of red light leads to the generation of a signal on which halobacteria begin to move toward the light source. By the absorption of a photon of blue light, it is occurred the opposite reaction. The maximum optical effect is achieved in both cases at wavelengths of $\lambda = 565$ and $\lambda = 370$ nm, respectively (Mosin et al., 2014). Thus, the photosensor reaction provides the optimal for the cell spatial orientation. Cells leave areas, which penetrates detrimental shortwave solar radiation and by means of flagella or gas vacuoles are concentrated in a favorable light condition area. This mechanism provides optimal conditions for the growth and vital function of halobacteria. Furthermore, the cell membrane of halovacteria contains another membrane protein, halorhodopsin serving as a light-dependent pump of chlorine ions ($Cl^-$), the main function of which is the transport of $Cl^-$ into the cell (Mosin et al., 2013b). Life in environments with high concentrations of NaCl has resulted in the development in halobacteria an effective system of active transport of $Na^+$ and $K^+$, whereby $Na^+$ is pumped out the cell and $K^+$, on the contrary, is pumped into the cell. As a result, the $Na^+$ content in the cytoplasm is maintained at a low level.
Despite the similarity of the mechanism of action of BR with the visual animal protein – rhodopsin, the amino acid sequence of BR differs from the animal rhodopsin that suggests their independent evolutionary origins. This is confirmed by the fact that the BR molecule forms 13-*cis, trans*- configuration rather than 11-*cis, trans*-configuration as in the animal rhodopsin (Lanyi, 1998). However, the conformation of BR indicates that the protein belongs along with the rhodopsin to the family of transport G-proteins that involved in a large number of biochemical signal processes in the cell.
According to the structure and location in the cell membrane BR refers to integral transmembrane proteins, penetrating the entire thickness of the cell membrane of halophiles, which is divided into three main fractions: yellow, red and purple. The purple fraction, containing 75 % of BR, carotenoid and phospholipid (mainly phospho glycerol with a small amount of non-polar lipids and isoprenoids) and water forms natural two-dimensional crystals that can be investigated with using electron microscopy techniques and diffraction analysis – X-ray scattering and the scattering of electrons and neutrons on the surface of PM crystals (Lanyi, 2004).

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These methods proved the existence in the BR molecule 7 α-helical protein segments in the middle of which is symmetrically located the chromophore moiety as a retinal residue (Figure 2).

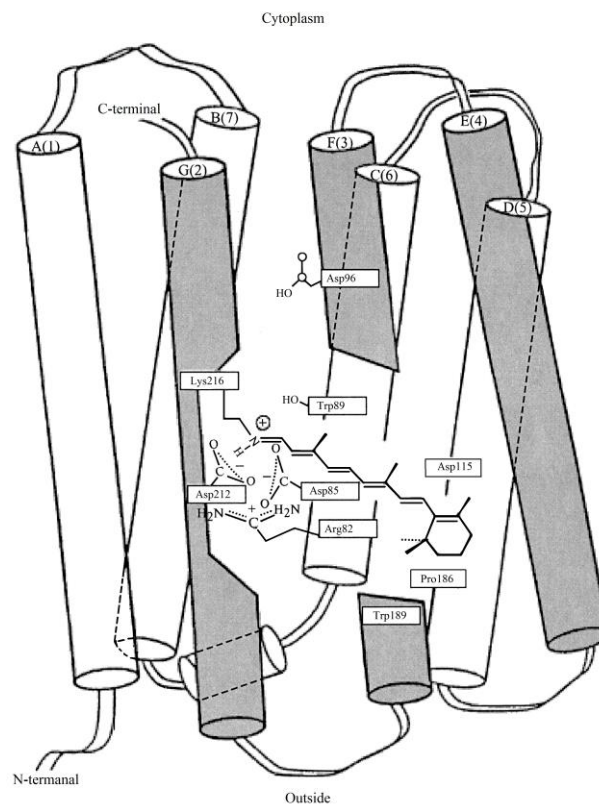


*Figure 2*: Location of the protein moiety of the BR and the retinal residue in the cell membrane of halobacteria *Halobacterium halobium* according to computer simulation: protein fragments of the BR molecules in the form of 7 penetrating the cell membrane α-helical segments are indicated in Latin characters; dark color designated the segments responsible for binding the retinal residue to the protein part of the BR molecule.

 

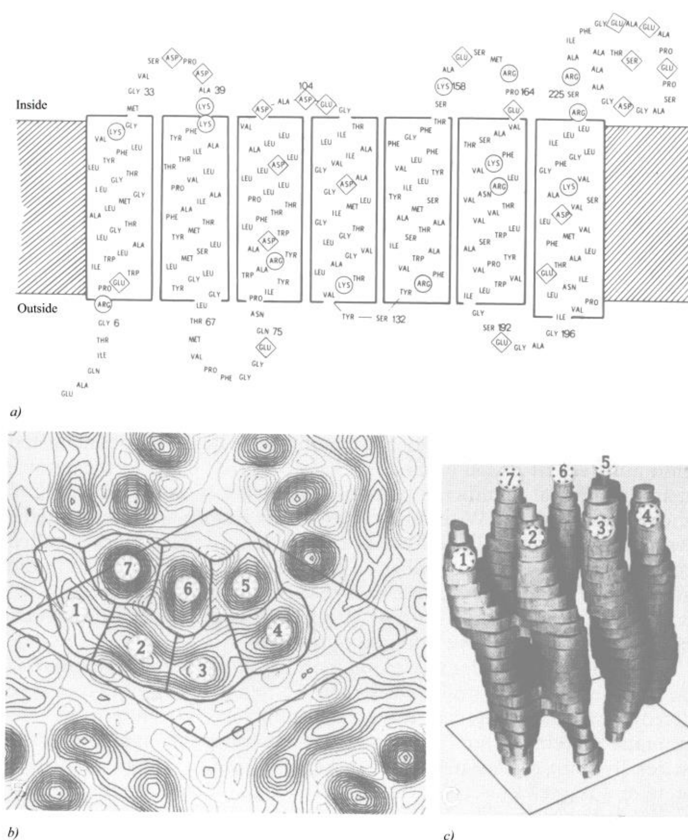


*Figure* 3: The structure of the BR molecule according to diffraction analysis:

a) – the primary structure of the BR molecule: amino acids indicated in Latin characters, circles and rhombs show the functionally important amino acids responsible for spatial orientation of α-helical segments of the protein moiety of the BR molecule and the formation of channels for the transfer of protons $H^+$ across the cell membrane;

b) – electron density map of PM (a single molecule of the protein is encircled in the center). Numbers 1–7 are designated α-helical segments of the BR molecule: – 1– A-segment; 2 –B-segment; 3 – C-segment; 4 – D-segment; 5 – E-segment; 6 – F-segment; 7 – G-segment;

c) – the spatial structure of the BR molecule: 1 – A-segment; 2 – B-segment; 3 – C-segment; 4 – D-segment; 5 – E-segment; 6 – F-segment; 7 – G-segment.

The polypeptide chain of BR consists of 248 amino acid residues, 67 % of which are hydrophobic (Jap et al., 1983), while 33 % – hydrophilic residues formed by aspartic and glutamic acids, arginine and lysine (Figure 3a). These residues play an important structural and functional role in the spatial orientation of α-helical segments of the BR molecule, which is organized in the purple membrane in an orderly manner in the form of trimmers with an average diameter of about 0.5 mm and a thickness of 5–6 nm. Each trimmer is surrounded by six others so that a proper hexagonal crystal lattice is formed (Figure 3b). The individual BR molecule consists of 7 α-helix segments arranged in the direction perpendicular to the plane of the cytoplasmic membrane (Figure 3c). Hydrophobic domains represent transmembrane segments, whereas hydrophilic domains protrude from the membrane and connect the individual intra membranous α-helical segments of protein segments in the BR molecule (Grigorieff, 1996).

***The mechanism of functioning of BR***

BR acts as a light-dependent proton pump, pumping protons across the cell membrane and generates an electrochemical gradient of $H^+$ on the surface of the cell membrane, which energy is used by the cell for the synthesis of ATP in the anaerobic photosynthetic phosphorylation (Haupts et al., 1997). The mechanism of ATP synthesis is called "non-chlorophyll photosynthesis", in contrast to the plant photosynthesis with the participation of chlorophyll. In this mechanism, at absorption of a light photon the BR molecule becomes decolorized by entering into the cycle of photochemical reactions, resulting in the release of a proton to the

outside of the membrane, and the absorption of proton from intracellular space. The formation of concentration gradient of $H^+$ leads to the fact that the illuminated halobacteria cells begin to synthesize ATP, i.e. convert light energy into the energy of chemical bonds. Due to this, the pH value inside the cytoplasm keeps the constant value – about 3 units and only slightly dependent on the pH of the exterior medium, which can reach 10–12 units.

The mechanism of subsequent sequential proton transfer of $H^+$ with the participation of the BR molecule across the cell membrane includes a chain of hydrogen bonds formed by the side residues of hydrophilic amino acids extending through the entire thickness of the protein. This $H^+$ proton transfer through the protein chain is carried out providing the protein consists of two parts and contains a photochrome functional group capable under the influence of light to change its microenvironment and thereby sequentially "lock" and "unlock" sites of binding of $H^+$ and its further transfer across the cell membrane. The role of such a "shuttle" mechanism between two conductors of $H^+$, one of which communicates with the exterior, and the other – with the cytoplasmic surface of the cell membrane plays a retinal residue linked by the aldimine bond (as in the visual pigments of animals) with a lysine-216 residue of the protein. Retinal has a 13-*trans* conformation and is located in the membrane tunnel between protein α-segments of the BR molecule, blocking the flow of protons. By the absorption of a light photon it occurs reversible isomerization of 13-*tras*-BR ($\lambda_{max}$ = 548 nm) (the quantum yield 0.03 at t = 20 $^0$C) into the 13-*cis*-BR ($\lambda_{max}$ = 568 nm) (Zimanyi et al., 1993), initiating a cascade of photochemical reactions lasting from 3 ms to 1 ps with the formation of transitional intermediates J, K, L, M, N, and O, followed by separation of $H^+$ from the retinal residue of BR and the connection of $H^+$ from the side of cytoplasm (Figure 4). In this process, the retinal residue is specifically bent in the membrane tunnel forming the transmembrane transport $H^+$ channel from the cytoplasm to the outside environment, and carries a proton $H^+$ from the inner cytoplasmic membrane to the outer membrane of the cell. In this case, a proton $H^+$ from the retinal residue is transferred to the Asp-85-residue, after that the resulting vacancy is filled with a proton $H^+$ transferred from the residue Asp-96. As a result, between the internal and external surface of the membrane forms a concentration gradient of $H^+$, which leads that illuminated cells begin to synthesize ATP, i.e. convert light energy into energy of chemical bonds. This process is reversible and in the dark flows in the opposite direction. In this way the BR molecule behaves as a photochromic carrier with a short relaxation time − the transition from the excited state to the ground state. Optical characteristics of BR vary depending on the method of preparation of PM embedded onto the polymer matrix.

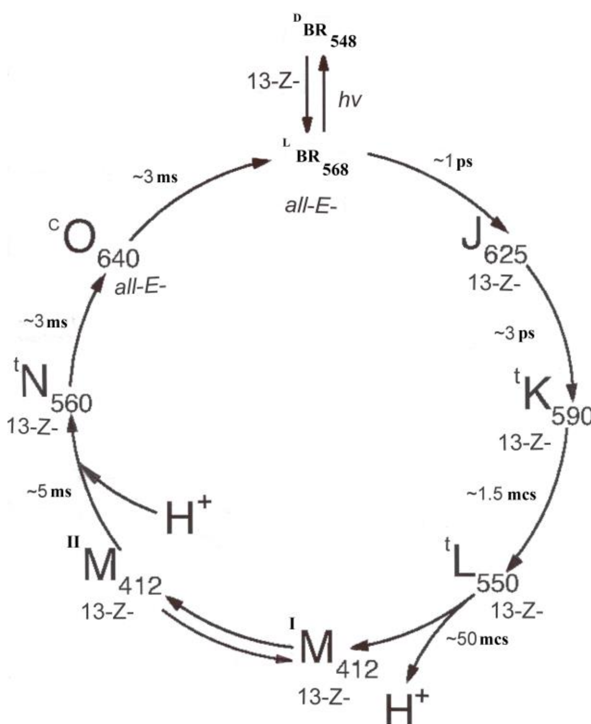


*Figure* 4: Photocycle scheme of BR (aqueous solution, pH = 7.2; t = 20 $^0$C). Latin numbers J, K, L, M, N, O denote spectral intermediates of BR. $^I$M and $^{II}$M represent spectral intermediants of *meta*- bacteriorhodopsin with the protonated ($^I$M) and deprotonated ($^{II}$M) aldimine bond. L and D denote dark and light forms of pigments. The subscripts correspond to the position of the absorption maximum in the photocycle intermediates (nm)

An interesting feature of the metabolism of halobacteria is at that the presence of oxygen and the organic compound (amino acids, peptones), which can be used as growth substrates and sources of energy, halophiles can grow in the dark, by switching on a heterotrophic photosynthetic metabolism (Mosin & Ignatov, 2014b). However, with a lack or even in the complete absence of oxygen and under the bright light in the cell membrane of halobacteria is synthesized BR, allowing them to use solar energy for growth and ATP synthesis. Thus, halobacteria are capable to carry out the synthesis of ATP molecules as due to chemical energy released in the process of oxidation in the respiratory chain, as well as using the light energy absorbed by BR.

***The practical application of BR in bionanotechnology***

BR is the focus of bio-and nanotechnology because of its high sensitivity and resolution, and is used in molecular bioelectronics as natural photochromic material for light-controlled electrical regulated computer modules and optical systems (Vought & Birge, 1999). *In* addition, BR is very attractive as a model for studies related to the research into the functional activity and structural properties of photo-transforming membrane proteins embedded into the native and photo-converting membranes (Mosin et al., 1999).
Nanofilms produced using the BR-containing PM were first obtained and studied in this country in the framework of the project "Photochrome" (Mosin et al., 1994), when it was demonstrated effectiveness and prospects for the use of BR as photochromic material for holographic recording (Figure 5).
The main task for the manufacture of BR-containing nanofilms is the orientation of PM between the hydrophobic and hydrophilic media. Typically, to improve the characteristics of the BR-containing films use multiple layers of PM that are applied to the surface of the polymeric carrier and dried up, preserving their natural structure. The best results are achieved in the manufacture of nanofilms embedded onto the gelatin matrix (Shuguang et al., 1993). This allows achieve high concentration of BR (up to 50 %) in nanofilms and avoid aggregation of cell membrane fragments and destruction of BR in the manufacturing process (Weetall, 1996).

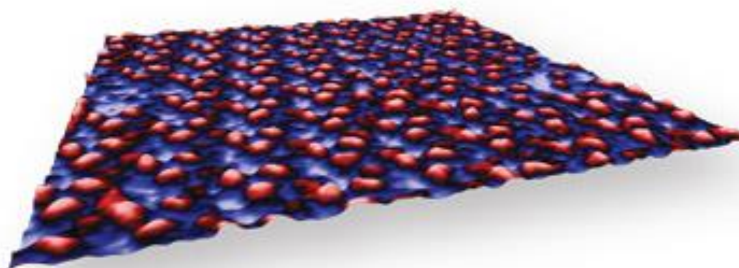

*Figure 5*: Artificial membrane based on the BR-containing PM in scanning electron microscope (SEM): scanning area – 100×100 mm; resolution – 50 nm; magnification – 100000 times. PM shown in purple, BR – in red color.

Being embedded into a gelatin matrix BR-containing PM fragments are durable (~$10^4$ h) and resistant to solar light, the effects of oxygen, temperatures greater than 80 ℃ (in water) and up to 140 ℃ ( in air), pH = 1–12, and action of most proteases (Downie et al., 1998). The dried PM are stacked on top of each other, focusing in the plane of the matrix, so that a layer with 1 μm thickness contains about 200 monolayers (Korposh et al., 2005). When being illuminated such nanofilms exert the electric potential at 100–200 mV, which coincides with the membrane potential of living cells (Seitz & Hampp, 2000). These factors are of great practical importance for integration of PM into polymeric nanomatrix with keeping photochemical properties.

***Biosynthesis of BR***

The technology of preparation of BR consists in growing of halobacteria on liquid artificial synthetic media with 15–20 % NaCl, containing synthetic amino acids or on natural media with peptones or protein-vitamin concentrate (PVC) of yeast (Mosin & Ignatov, 2014c).
Artificial synthetic media are represented by mixtures of synthetic essential amino acids (glycine, alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine,

proline, serine, threonine, tyrosine, tryptophan, valine), nucleotides (adenosine-5-monophosphate, uridine-5-monophosphate), inorganic salts and vitamins.

Peptones – are products of partial hydrolysis of animal proteins (milk, meat), consisting of mixtures of different polypeptides; also contain di- and tripeptides, and free amino acids. Peptones are formed by the action of proteolytic enzymes of the gastric and pancreatic juices (pepsin, trypsin) on natural proteins, as well as by mild hydrolysis of animal proteins by acids and alkalis. The source of the peptone protein depends on the species from which it is derived: meat, fish, egg peptones etc.

PVC is a dry biomass of feed yeast of saccharomyces genus of *Saccharomyces cerevisiae*, grown on hydrocarbons – oil parafins (paprin) or natural gas (gaprin). It contains approx. 50 % of protein, a full set of vitamins, a large number of trace elements (iron, manganese, iodine, magnesium, sodium, zinc) and amino acids.

Depending on the needs of a particular type of halobacteria in sources of growth substrates use artificial synthetic nutrient media or media prepared on the basis of natural peptons of PVC of yeast.

For the biosynthesis of BR often use the extreme aerobic photo-organotrophic halobacterium *Halobacterium halobium* (Mosin et al., 2014). This bacterium is grown on the synthetic liquid complex medium containing 18 amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, valine ), nucleotides (adenosine-5-monophosphate, uridine-5-monophosphate), inorganic salts of sodium, magnesium, manganese, copper, calcium, zinc, iron, potassium, ammonium, phosphorus) and biotin, folic acid and vitamin $B_{12}$.

The process of growing the halobacteria is conducted on an orbital shaker in flat-bottomed flasks in condition of intensive aeration under the light of monochrome fluorescent lamps. Bacterial growth was measured by optical density of the cell suspension at $\lambda$=620 nm on a spectrophotometer. As is shown in Figure 6, under optimal growing conditions (incubation period 4–5 days, temperature t = 35 $^0$C, illumination with monochromatic light at $\lambda$ = 560 nm) in cells is synthesized the purple carotenoid pigment, characterized as BR by the spectral ratio of protein and chromophore fragments $D_{280}/D_{568}$ = 1.5:1.0 in the molecule (Mosin & Ignatov, 2014b). The subsequent isolation of BR from the PM fraction is carried out by a combination of physical, chemical and enzymatic methods (Mosin & Ignatov, 2014d).

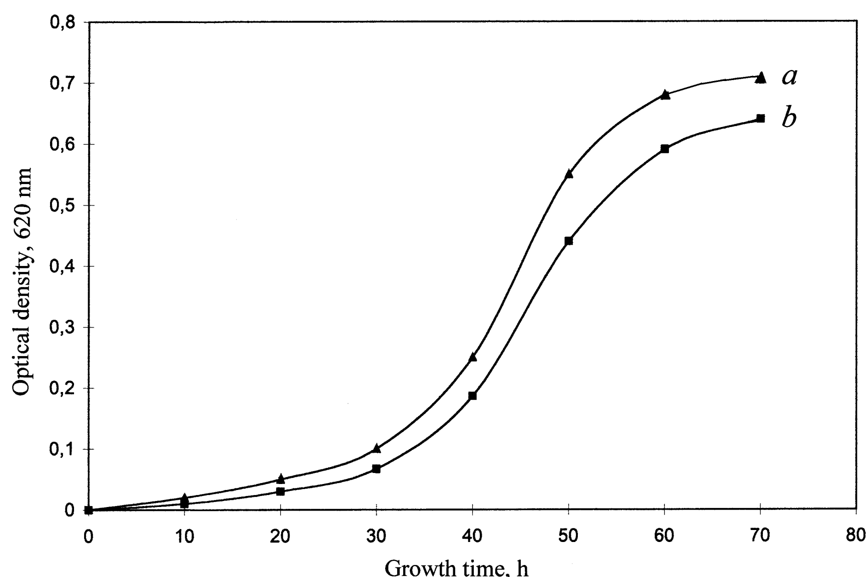


*Figure 6*: Growth dynamics of *H. halobium* under different conditions: *a)* – the peptone medium; *b)* – complex synthetic medium. Growing conditions: incubation period of 4–5 days at t = 35 $^0$C, illuminating by monochromatic light with a wavelength of $\lambda$ = 560 nm.

### *Isolation of BR*

Isolation and purification of BR from PM fraction is carried out with using a light-shielding lamp equipped with an orange color filter as BR is very sensitive to light and light isomerization.

The main stages for obtaining BR are:

- Growing the halobacterium *H. halobium* on artificial or natural nutrient media;
- Cell disruption and lysis of cell walls;
- Allocation fraction of PM;
- Cleaning of PM from low- and high molecular weight impurities, cellular RNA and carotenoids;

• Dissolving the PM fraction in a 0.5 % solution of the ionic detergent, sodium dodecyl sulfate (SDS-Na) to form a microemulsion;
• Precipitation of BR from the microemulsion by methanol;
• Gel Permeation Chromatography (GPC) on Sephadex G-200;
• Electrophoresis in 12.5 % polyacrylamide (PAGE) gel.

The protein is localized in the PM; the release of low molecular weight impurities and intracellular contents was reached by osmotic shock of cells with distilled water in the cold after the removal of 4.3 *M* NaCl and the subsequent destruction of the cell membrane by ultrasound at 22 kHz. For the destruction of cellular RNA the cellular homogenate was treated with Rnase I. PM fraction along with the desired protein in a complex with lipids and polysaccharides also contained impurity of related carotenoids and proteins. Therefore, it was necessary to use special methods of fractionation of the protein without damaging its native structure and dissociation. That required applying the special methods of purification of carotenoids and lipids, and the subsequent GPC on Sephadex G-200. Removing of carotenoids, consisting in repeated treatment of PM with 50 % (v/v) EtOH at t = 4 $^0$C, was a routine but necessary step, in spite of the significant loss of chromoprotein. It was used five treatments by 50 % (v/v) EtOH to obtain the absorption spectrum of purified from carotenoids PM suspension (4) and (5) (the degree of chromatographic purity of 80−85 %), as shown in Figure 7 at various processing stages (*b*) and (*c*) relative to the native BR as a control (*a*). The formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption spectrum of PM (Figure 7*c*) – the main bandwith (1) with the absorption maximum at $\lambda$ = 568 nm is caused by the light isomerization of the chromophore by the C13=C14 bond is determined by the presence of 13-*trans*-retinal residue in $BR_{568}$; additional low-intensity bandwith (2) at $\lambda$ = 412 nm characterizes a minor impurity of a spectral form of *met*a-bacteriorhodopsin $M_{412}$ (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwith (3) at $\lambda$ = 280 nm is determined by the absorption of aromatic amino acids in the polypeptide chain of the protein (for the native BR – $D_{280}/D_{568}$ = 1.5:1.0).

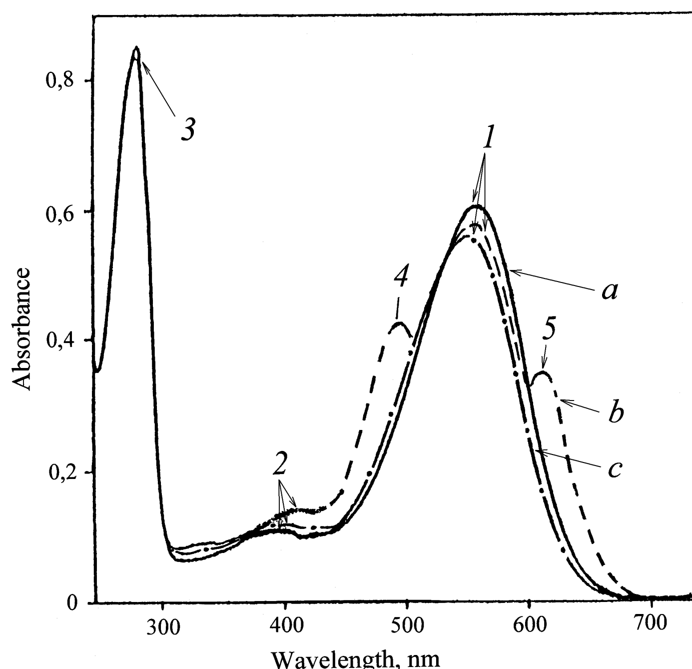


*Figure 7*: The absorption spectra of the PM (50 % (v/v) EtOH) at various stages of processing: (*a*) – the natural BR (control); (*b*) – PM after intermediate treatment; (*c*) – PM purified from carotenoids. The bandwith (1) is the spectral form of $BR_{568}$, (2) – impurity of spectral form of *meta*-bacteriorhodopsin $M_{412}$, (3) – the total absorption bandwith of aromatic amino acids, (4) and (5) – extraneous carotenoids. As a control used the native BR

***The fractionation and chromatographic purification of BR***

The fractionation and chromatographic purification of the protein was the next necessary step of the research. As BR, being an integral membrane protein intricately penetrates bi-lipid layer in form of seven $\alpha$-helices, the use of ammonium sulfate and other conventional agents to salting out did not give a positive result for isolation of the protein. The resolving was in the translation of the protein to a soluble form by the colloidal dissolution

(solubilization) in an ionic detergent. Using as the ionic detergent SDS-Na was dictated by the need to carry out the solubilization of the protein in a native, biologically active form in complex with 13-*trans*-retinal, because BR solubilized in 0,5 % (v/v) SDS-Na retains a native α-helical configuration (Mosin & Ignatov, 2014e). Therefore, there is no need to use organic solvents as acetone, methanol and chloroform for purification of lipids and protein, and precipitation and delipidization is combined into a single step, which significantly simplifies the further fractionation. A significant advantage of this method is that the isolated protein in complex with lipids and detergent molecules was distributed in the supernatant, and other high molecular weight impurities – in unreacted precipitate, easily separated by centrifugation (Mosin & Ignatov, 2014f). Fractionation of the protein solubilized in a 0.5% (w/v) SDS-Na and its subsequent isolation in crystalline form was achieved at t = 4 $^0$C in three steps precipitating procedure with treatment by methanol, reducing the concentration of detergent from 0.5; 0.25 and 0.1 % (w/v) respectively.

The final stage of BR purification involved the separation of the protein from low-molecular-weight impurities by using GPC-method. For this purpose the BR containing fraction was passed twice through a chromatography column with dextran Sephadex G-200 balanced with 0.09 *M* Tris-buffer (pH = 8.35) containing 0.1 % (w/v) SDS-Na and 2.5 *mM* EDTA. The elution was carried out at t = 20±25 $^0$C with 1 mM Tris-HCl buffer (pH = 7.6) at rate of 10 ml/cm$^{2}$·h. The data on purification of BR of phospholipids and carotenoids are shown in Table 2. As it is demonstrated in Table 2, 84 % of phospholipids was removed by five washes (65, 70 and 76 % was removed by 1st, 2nd and 3nd wash respectively). The total endogenous phospolipid removal on the BR peak was 92 % relative to the native PM.

*Table 2*: Summary results for the isolation and purification of BR by various methods

| Sample | PM content, mol PM/mol BR | Phospholipid and carotenoid removal, % | BR yield*, % |
|---|---|---|---|
| PM fraction | 20,5 | – | – |
| PM washed with EtOH | | | |
| 1 wash | 16.9 | 65 | 93 |
| 2 wash | 15.1 | 70 | 90 |
| 3 wash | 14.5 | 76 | 88 |
| 4 wash | 13.6 | 81 | 84 |
| 5 wash | 13.2 | 84 | 80 |
| BR crystallised from MeOH | 12.9 | 86 | 75 |
| BR from GPC on Sephadex G-200 | 10.2 | 92 | 86 |

**Notes:**

* Percentage yield is indicated in mass.% relative to BR solubilized in 0.5 % SDS-Na before concentration.

## Conclusions

Haobacteria is a taxonomic group of extreme aerobic microorganisms having great practical bionanotechnological potential. BR synthesized by these microorganisms is the focus of bio-and nanotechnology because of its high sensitivity and resolution, and may be used in molecular bioelectronics as natural photochromic material for light-controlled electrical regulated computer modules and optical systems. The technology of BR biosynthesis allows obtain milligram quantities of pure crystal protein. The main advantage is that BR retains its natural configuration and the ability to undergo photochemical transformations. By this method is possible to obtain similar to BR transmembrane proteins of halobacteria – sensorodopsin and halorodopsin. The unique properties of these natural bacteriorhodopsins provide a wide range of optical applications in which they can be applied, because the integration of these proteins in the most advanced technical optical systems is very simple.

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