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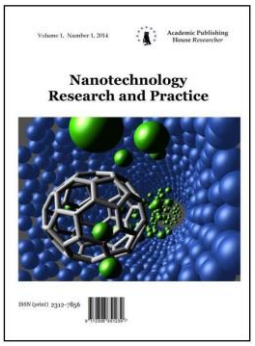

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New Nano- and Biotechnological Applications of Bacterial and Animal Photoreceptor Pigments – Bacteriorhodopsin, Rhodopsin and Iodopsin

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Abstract

This paper views predominately the structure and function of animal and bacterial photoreceptor pigments (rhodopsin, iodopsin, bacteriorhodopsin) and new aspects of their nano- and biotechnological usage. On an example of bacteriorhodopsin was described the method of its isolation from purple membranes of photo-organotrophic halobacterium *Halobacterium halobium* by cellular autolysis by distilled water, processing of bacterial biomass by ultrasound at 22 KHz, alcohol extraction of low and high-weight molecular impurities, cellular RNA, carotenoids and lipids, the solubilization with 0,5 % (w/v) SDS-Na and subsequent fractionation by methanol and gel filtration chromatography on Sephadex G-200 Column balanced with 0.09 M Tris-HCl buffer (*pH* = 6,76) with 0,1 % (w/v) SDS-Na and 2,5 mM EDTA. Within the framework of the research the mechanism of color perception by the visual analyzer having the ability to analyze certain ranges of the optical spectrum, as colors was studied along with an analysis of the additive mixing of two colors. It was shown that at the mixing of electromagnetic waves with different wavelengths, the visual analyzer perceive them as separate or average wave length corresponding to mix color.

Keywords: vision, rhodopsin, iodopsin, bacteriorhodopsin, additive color mixing.

1. Introduction

Vision (visual perception) is a process of psycho-physiological processing of the images of surrounding objects, carried out by the visual system, which allows get an idea of the size, shape and color of surrounding objects, their relative position and the distance between them. By means of this animals can receive 90 % of all incoming information to the brain.

The function of the visual system is carried out through various interrelated complex structures designated as visual analyzer, consisting of a peripheral part (retina, optic nerve, optic tract) and the central department of combining stem and subcortical centers of the midbrain, as

well as the visual cortex of the cerebral hemispheres. The human eye can perceive only light waves of a certain length – from λ = 380 to λ = 770 nm.

Light rays from treated subjects pass through the optical system of the eye (cornea, lens and vitreous body) and onto the retina, where the light-sensitive photoreceptor cells (rods and cones) are located. Light incidented on the photoreceptors, triggers a cascade of biochemical reactions of visual pigments (in particular, the most studied of them is rhodopsin responsible for the perception of electromagnetic radiation in the visible range), and in its turn – the occurrence of nerve impulses, which are transmitted through the following retinal neurons and further to the optic nerve. The optic nerve carries the nerve impulses into the lateral geniculate body – the subcortical center of vision, and thence to the cortical center, located in the occipital lobe of the brain, where the visual image is formed.

Over the last decade has been obtained new data revealing the molecular basis of visual perception. There were identified visual molecules of eucariotes (rhodopsin, iodopsin) and procariots (bacteriorhodopsin) involved in light perception and was cleared up the mechanism of their action.

The structural research of rhodopsin and its affiliated chromophore proteins (iodopsin, bacteriorhodopsin) and the analysis of their functions have been carried out in the Scientific Research Center of Medical Biophysics (Bulgaria) throughout the last 20 years. The purpose of the research was the studying of basic biomolecular mechanisms associated with visual perception and some nano- and biotechnological applications of visual phototransforming pigments as trans-membrane protein bacteriorhodopsin, extracted from purple membranes of a halophilic bacterium *Halobacterium halobium*.

2. Material and methods

As a producer of bacteriorhodopsin (BR) was used a carotenoid strain of extreme photo-organorotrophic 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. 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; $MgSO_4 \cdot 7H_2O$ – 20,0; KCl – 2,0; 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 °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. 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 °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 °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 °C. Then 10 ml of the same buffer was added and kept for 10–12 h at 4 °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 °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} (ε_{280} = $1{,}1 \cdot 10^5$; ε_{568} = $6{,}3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [1]. PM regeneration is performed as described in [2]. Yield of PM fraction – 120 mg (80–85%). The fraction of 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 °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 °C. The reaction mixture was kept for 14–15 h in ice bath at 4 °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). 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. Sephadex) equilibrated with buffer containing 0,1 % (w/v) SDS-Na and 2,5 mM ETDA. Elution proceeded by 0,09 M Tris-HCl containing 0,5 M NaCl, *pH* = 6,76 at a flow rate of 10 ml/cm^2 · h. Combined protein fraction was subjected to freeze-drying, in sealed glass ampoules (10 × 50 mm) and stored in frost camera at -10 °C.

Quantitative analysis of the protein 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 of pigments were recorded on programmed DU-6 spectrophotometer ("Beckman Coulter", USA) at λ = 280 nm and λ = 750 nm.

IR-spectra were registered on Brucker Vertex IR spectrometer ("Brucker", Germany) (a spectral range: average IR – 370–7800 cm^{-1}; visible – 2500–8000 cm^{-1}; the permission – 0,5 cm^{-1}; accuracy of wave number – 0,1 cm^{-1} on 2000 cm^{-1}) and Thermo Nicolet Avatar 360 Fourier-transform IR.

Colors were analyzed by using color analyzer "Tsvetan" ("Photopribor", Cherkassk, Ukraine). Operating relative absorbance, % – from -80 to 70. Measurement error, ±5 %. Response time from 0,4 to 63 sec. Overall dimensions, 300 mm.

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.

3. Results and discussion

3.1. Theoretical aspects of molecular basis of vision

The process of perception of light has a definite localization in photoreceptor light-sensitive cells of the retina. The retina in its structure is a multilayer layer of nervous tissue that is sensitive to light, which lines the inside of the back of the eyeball. The pigmented retina located at the membrane is referred to as retinal pigmented epithelium (RPE), which absorbs light passing through the retina. This prevents the reverse reflection of the light through the retina and does not allow the vision to disperse.

Light enters through the eye and creates a complex biochemical reaction in the photoreceptor cells of the retina. Photoreceptor cells are divided into two types that due to their characteristic form are designated as rods and cones [3]. Rods are receptors of light of low intensity; they arranged in a colored layer of the retina, in which is synthesized photochromic protein rhodopsin, responsible for color perception. Cones on the contrary contain a group of visual pigments (iodopsin), and adapted to distinguish different colors. Rods can persive black and white images in the dim light, cones – to carry out color vision in bright light. Human retina contains approximately 3 million of cones and 100 million of rods. Their dimensions are very small – the length of about 50 mm, the diameter from 1 to 4 μm.

The electrical signals generated by the rods and cones, are handled by other retinal cells – bipolar and ganglion cells before they are transmitted to the brain via the optic nerve [4]. Additionally, there are two intermediate layers of neurons. Horizontal cells transmit messages back and forth between the photoreceptor cells, bipolar cells and each other. Amacrine cells of the retina are linked to bipolar cells, ganglion cells, as well as with each other. Both types of these intermediate neurons play a major role in the processing of visual information at the level of the retina before it is transmitted to the brain for final processing.

Cones are approximately 100 times less sensitive to light than rods, but much better perceive the rapid movement. The wand can be stimulated by a single photon. A cascade of molecular interactions enhances this "quantum" of information into a chemical signal, which is then perceived by the nervous system. The degree of enhancement of the signal varies depending on ambient light: the rods are more sensitive under low light than under bright light. As a result, they operate effectively in a wide range of ambient light. The sensory system of rods is packed up in clearly distinguishable cellular substructure that can be easily selected and investigated *in vitro* in isolated state. This property makes them as indispensable object for further structural-functional studies as well as the studies of photoreceptor pigments (rhodopsin, iodopsin). These animal photoreceptor pigments are used as comparative models for studying of bacterial photoreceptor pigment bacteriorhodopsin (BR) isolated from purple membranes of halobacterium *Halobacterium halobium*.

3.2. Rhodopsin and its structural and functional properties

Rhodopsin [5] is one of the most important integral photoreceptor proteins of rod cells, which absorbs a photon and creates a biochemical response constituting a first step in a chain of events that provide vision. Rhodopsin consists of two components – a colorless protein opsin and a chromophore component 11-*cis*-retinal residue, acted as the light acceptor (Fig. 1). The absorption of a light photon by 11-*cis*-retinal "turns on" the enzymatic activity of opsin and further photosensitive biochemical cascade of reactions that are responsible for vision [6].

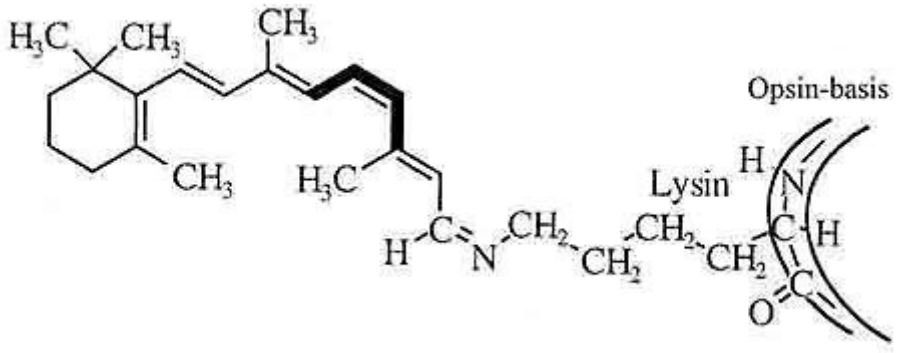

Fig. 1. Configuration of photosensitive chromophore of rhodopsin in the basic (unexcited) phase (at the double bond is marked 11-cis-configuration)

Rhodopsin belongs to the group of the G-protein-coupled receptors (GPCR-receptors) of the retilylidene protein family responsible for trans-membrane signaling mechanism based on the interaction with intracellular membrane G-proteins – universal intermediaries in the transmission of hormonal signals from the cell membrane receptors to effector proteins, causing the final cellular response. The establishment of the spatial structure of rhodopsin is so important because rhodopsin as the "originator" of the family of GPCR-receptors is a "model" for the structure and function of other receptors that it is extremely important from fundamental scientific and practical points of view [7].

The spatial structure of rhodopsin was long defied by the study of "direct" methods – X-ray diffraction and NMR spectroscopy, while the molecular structure of related to rhodopsin trans-membrane chromoprotein bacteriorhodopsin [8] having a similar structure, performing the functions of ATP-dependent translocase in the cell membranes of halophilic microorganisms pumping protons across the cytoplasmic membrane of the cell and is involved in the anaerobic photosynthetic phosphorylation (non-green synthesis), was determined as early as 1990. On the contrary the structure of rhodopsin remained unknown until 2003 [9]. The opsin fragment of the rhodopsin molecule has 348 amino acid residues in a polypeptide chain that is formed by seven transmembrane α-helix segments situated across the membrane and joined with short non-helix sections [10]. The N-terminus of α-helix is located in the extracellular region, while the C-terminus – in the cytoplasmic region. The 11-*cis*-retinal residue is connected to one of the α-helixes, located

near the middle of the membrane, so that its long axis is parallel to the membrane surface (Fig. 2). It was also determined the dislocation of 11-*cis*-retinal aldimine bond with ε-amino group of Lys-296 residue located in the seventh α-helix.

Thus, 11-*cis*-retinal is mounted in the center of a complex highly organized protein in the cellular membrane comprising rods. This structure provides a photochemical "adjustment" of retinal residue, affecting its absorption spectrum. The free 11-*cis*-retinal in a dissolved form has an absorption maximum in the ultraviolet region – at a wavelength of 380 nm, while rhodopsin absorbs green light at 500 nm [11]. This shift in the wavelength of light is important from a functional point of view; it is aligned with the spectrum of light that enters the retina.

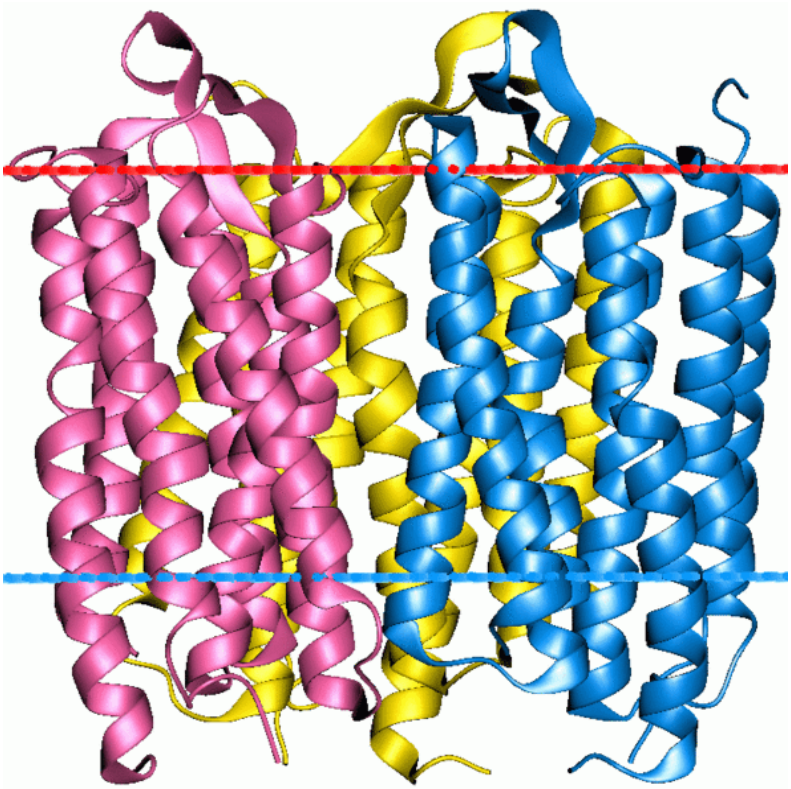

Fig. 2. The structure of rhodopsin according to computer modeling data

The absorption spectrum of rhodopsin is defined by properties of the chromophore – 11-*cis*-retinal residue and opsin fragment. This range in vertebrates has two characteristic peaks – one peak in the ultraviolet (λ = 278 nm) due to the opsin fragment, and the other – in the visible region (λ = 500 nm) corresponds to absorption of the chromophore (Fig. 3). Further transformation of rhodopsin under the action of light to the final stable product consists of a series of very fast intermediate stages. Investigating intermediates absorption spectra of rhodopsin in extracts at low temperatures at which these products are stable, allows describe in the detail the photochemical changes of rhodopsin [12].

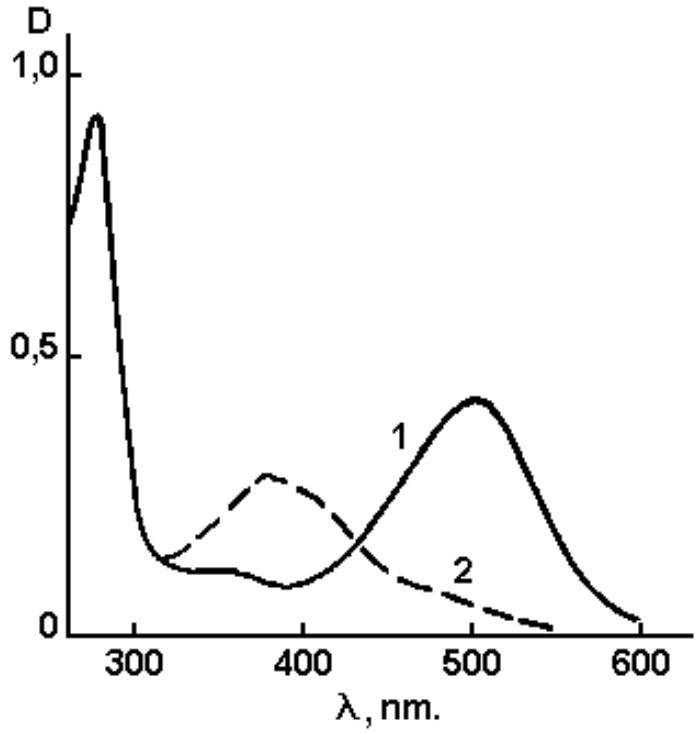

Fig. 3. Absorption spectrum of rhodopsin from the frog *Rana temporaria* (in water extract): 1 – rhodopsin (restored pigment); 2 – yellow indicator (discolored pigment)

Upon absorption of light photon it is occurred isomerization of 11-*cis*-retinal into 11-*trans*-retinal (quantum yield, 0,67), that induces a conformational change in the protein and activates photopsin and promotes its binding to G protein transducin, which triggers a second messenger cascade [13]. Subsequent cycles of the photochemical reactions of rhodopsin lead to a local depolarization of the membrane and the stimulation of the nerve impulse propagates along the nerve fiber due to changes in ion transport in the photoreceptor (Fig. 4). Subsequently rhodopsin restored (regenerated) with participation of retinal isomerase through the following steps: 11-*trans*-retinal → 11-*trans*-retinol → 11-*cis*-retinol → 11-*cis*-retinal, the latter is connected with opsin to form rhodopsin.

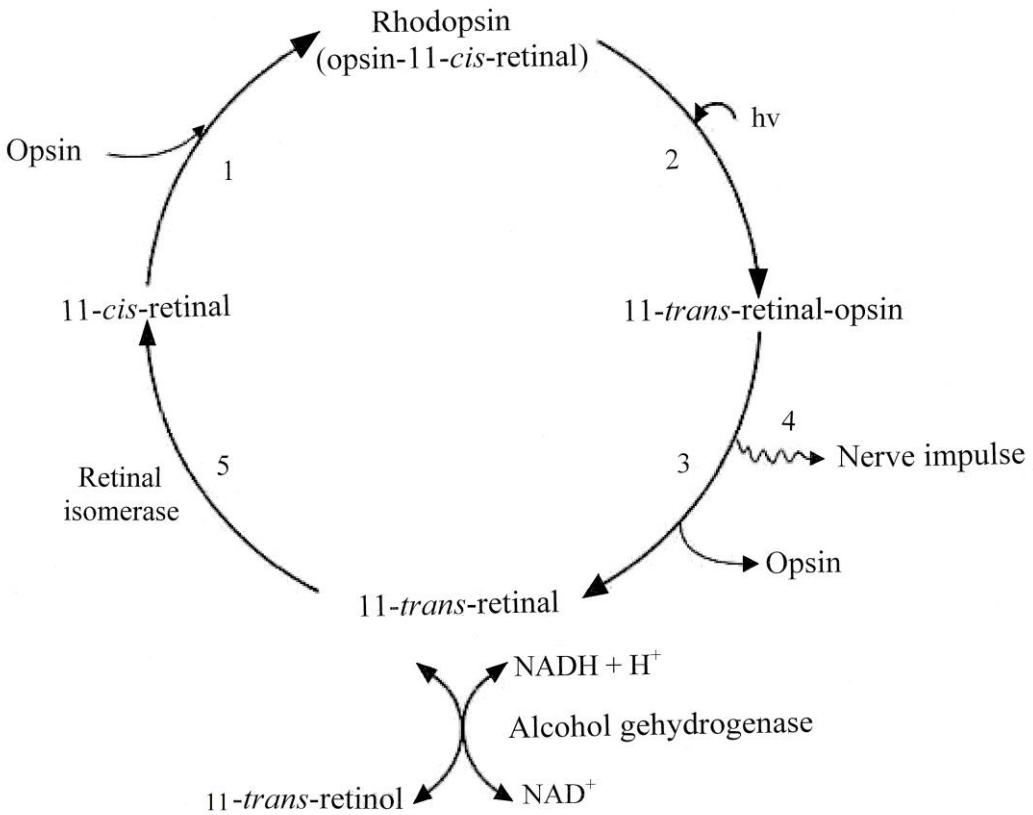

Fig. 4. A photocycle scheme of rhodopsin: 1 – 11-cis-retinal in the dark links with protein opsin to form rhodopsin; 2 – under light illumination occurs photoisomerization of 11-cis-retinal into 11-trans-retinal; 3 – 11-trans-retinal-opsin complex splits onto 11-trans-retinal and opsin; 4 – local depolarization of the membrane and the occurrence of a nerve impulse propagates along the nerve fiber; 5 – regeneration of the original pigment

3.3. Bacteriorhodopsin and its applications

Bacteriorhodopsin (BR), named by analogy to the visual apparatus of mammalian chromoprotein rhodopsin, was isolated from the cell membrane of extreme photo-organo-heterotrophic halobacteria *Halobacterium halobium* in 1971 by D. Osterhelt and W. Stohenius. This photo-transforming trans-membrane chromo-protein with the molecular weight ~26,5 kDa is a chromoprotein determining the purple-red culour of halophilic bacteria, contained as chromophore group an equimolar mixture of 13-*cis*-and 13-*trans*-retinol C20-carotenoid, bound by the Schiff base (as in the visual animal pigments) with Lys-216 residue of the protein.

In its structure and location in the cell membrane the BR refers to integral transmembrane proteins, penetrating the cell membrane, which is divided into three fractions: yellow, red and purple. The purple fraction comprising on 75% (w/w) of cell membrane (purple membranes or PM) consists from carotenoids, phospholipids (mostly phosphoglycerol diesters with a small amount of nonpolar lipids and isoprenoids) forms natural two-dimensional crystals which can be investigated using electron microscopy diffraction methods as X-ray scattering [15]. These methods have established the existence in the BR molecule seven α-helical protein segments, while in the middle are symmetrically located a retinal residue (Fig. 5).

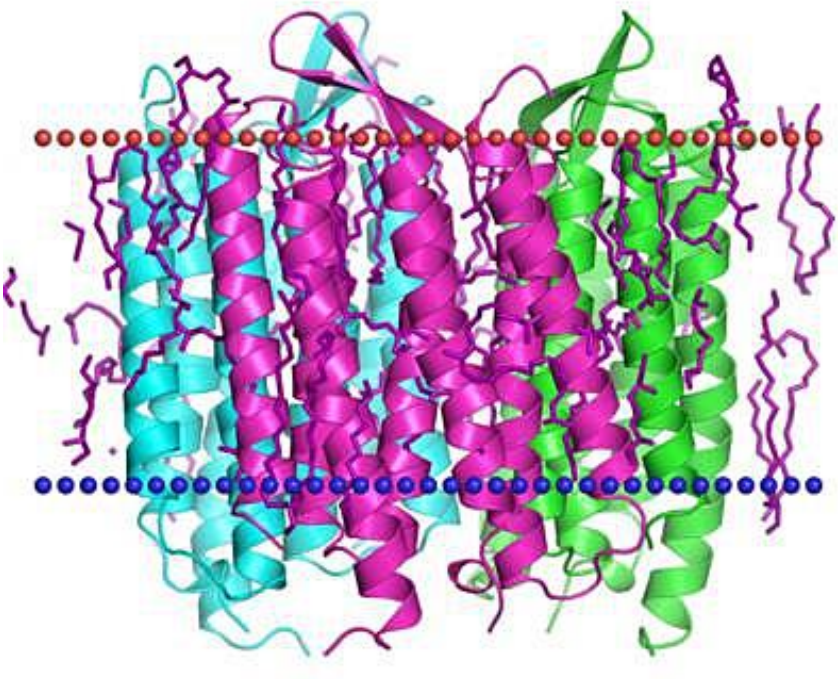

Fig. 5. The structure of BR from PM of halophilic bacterium *H. halobium* according to computer modeling data

The polypeptide chain of the BR molecule consists of 248 amino acid residues, 67% of which are hydrophobic, formed with the aromatic amino acids, and 33% – hydrophilic residues of aspartic and glutamic acids, arginine and lysine [16]. These residues play important structural and functional role in the spatial orientation of the α-helical segments of the BR molecule, arranged in PM in an orderly manner forming trimers with an average diameter ~0,5 μm and a thickness 5–6 nm; each trimmer is surrounded by six others so that to form a regular hexagonal lattice [17]. The BR molecule arranged in a direction perpendicular to the plane of the membrane. Hydrophobic domains represent transmembrane segments and hydrophilic domains protruding from the membrane connect the individual α-helical intramembranous segments of the BR molecule.

The BR acts as a light-dependent proton pump, pumping protons across the cell membrane that 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. 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 absorbtion of a light photon the BR molecule became 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. By the absorption of a light photon is occured reversible isomerization of 13-*tras*-BR (λ_{max} = 548 nm) (the quantum yield 0,03 at +20 °C) in the 13-*cis*-BR (λ_{max} = 568 nm) [18], 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 the BR and its connection from the side of cytoplasm (Fig. 6). As a result, between the internal and external surface of the membrane forms a concentration gradient of H^+, which leads that illuminated halobacteria cells begin to synthesize ATP, i.e. convert light energy into the energy of chemical bonds. This process is reversible and in the dark it 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 the BR molecule vary depending on the method of preparation of PM and the polymer matrix.

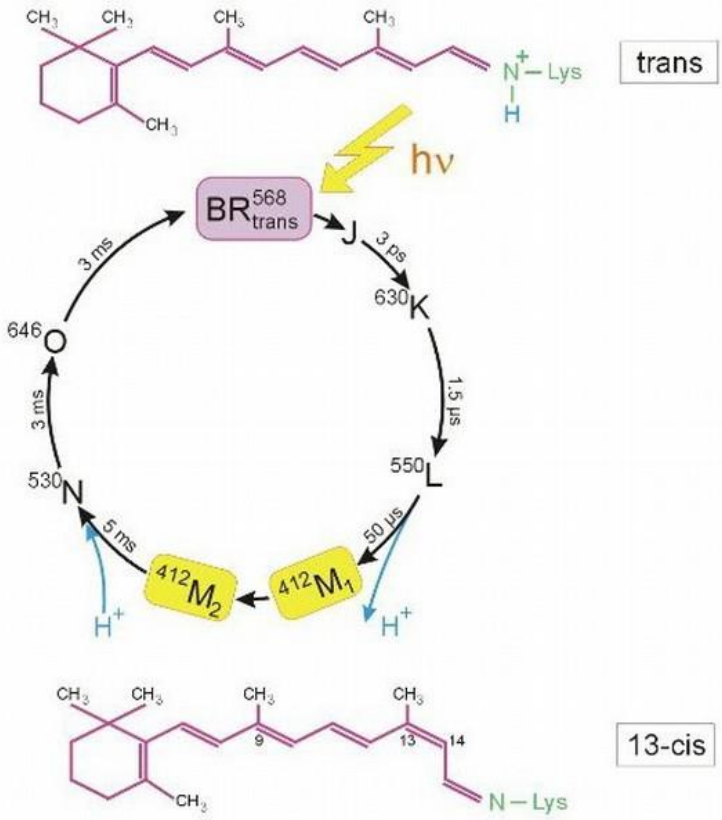

Fig. 6. A photocycle scheme of the BR (aqueous solution, pH = 7.2, t = +20 °C). Latin numbers J, K, L, M, N, O denote the spectral intermediates of BR. M_1 and M_2 represent spectral intermediants of meta- bacteriorhodopsin with the protonated and deprotonated aldimine bond. The superscripts correspond to the position of the absorption maximum of the photocycle intermediates (nm)

The 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 [19, 20]. In addition, the BR is very attractive as a model for studies related to the research of functional activity and structural properties of photo-transforming membrane proteins in the native and photo-converting membranes [21].

Nanofilms produced using the BR-containing purple membranes (PM) of halobacteria were first obtained and studied in this country in the framework of the project "Photochrome", when it was demonstrated effectiveness and prospects for the use of the BR as a photochromic material for holographic recording (Fig. 7). 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 based on gelatin matrix [22]. This allows achieve high concentration of the BR (up to 50 %) in nanofilms and avoid the aggregation of membrane fragments and destruction of the BR in the manufacturing process [23]. Embedded in a gelatin matrix PM fragments are durable (~10^4 h) and resistant to solar light, the effects of oxygen, temperatures greater than +80 °C (in water) and up to +140 °C (in air), *pH* = 1–12, and action of most proteases [24]. Dried PM is 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 [25]. When being illuminated

the nanofilms exert the electric potential 100–200 mV, which coincides with the membrane potential of living cells [26]. These factors are of great practical importance for integration of PM into polymeric nanomatrix with keeping photochemical properties.

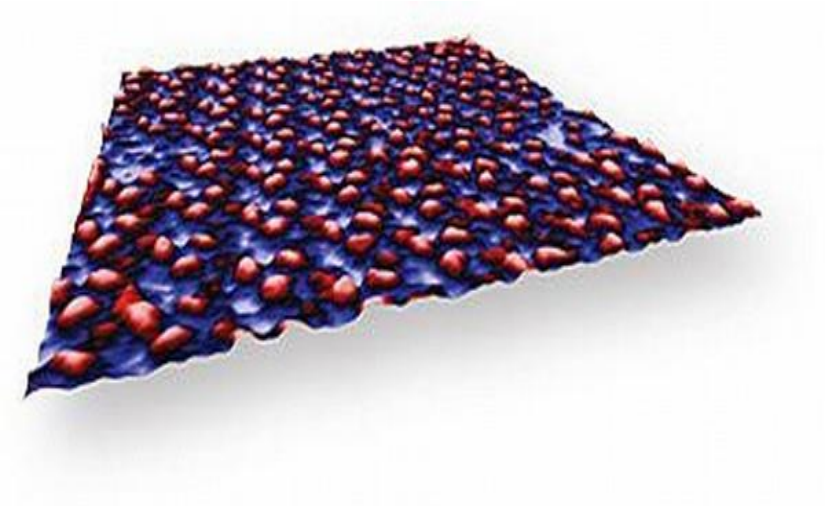

Fig. 7. An artificial membrane from the BR-containing PM in scanning electron microscope (SEM): scanning area – 100 × 100 mm, resolution – 50 nm, magnification – 100000 times. PM shown in purple, BR – in red color

The technology for preparation of the BR consists in growing of halobacteria on liquid synthetic growth media (with 15–20 % (w/w) NaCl) with amino acids, or on natural growth media with peptons – mixtures of polypeptides and amino acids derived from the partial hydrolysis product or powdered milk, animal meat by proteolytic enzymes (pepsin, trypsin, chymotrypsin), or protein- vitamin concentrate of yeast [27]. The subsequent isolation of the BR from purple membranes is carried out by a combination of physical, chemical and enzymatic methods [28]. Under optimal growing conditions (incubation period 4–5 days, temperature +35 ^{o}C, illumination with monochromatic light at λ = 560 nm) in cells are synthesized the purple carotenoid pigment, characterized as the BR by the spectral ratio of protein and chromophore fragments D_{280}/D_{568} = 1,5 : 1,0 in the molecule.

Within the framework of the research we described an effective method for isolation of the BR from PM fraction of photo-organotrophic halobacterium *H. halobium* consisted by cellular autolysis by distilled water, processing of bacterial biomass by ultrasound at 22 KHz, llocation of PM fraction, purification of PM from low and high-molecular weight impurities, cellular RNA, carotenoids and lipids, PM solubilization in 0,5 % (w/v) solution of the ionic detergent SDS-Na to form a microemulsion with the subsequent fractionation of the protein by methanol [29]. The protein is localized in PM; the release of low molecular weight impurities and intracellular contents is 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. The 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.

The BR being a trans-membrane protein intricately penetrates bilipid layer in form of seven α-helices, the use of ammonium sulfate and other conventional agents to salting out does 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 of solubilization of the protein in a native, biologically active form in complex with 13-*trans*-retinal, because the BR solubilized in 0,5 % (v/v) SDS-Na retains a native α-helical configuration [30]. 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 in 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. Fractionation of the solubilized in 0,5 % (w/v) SDS-Na protein and its subsequent isolation in crystalline form was achieved at 4 °C in three steps precipitating procedure with methanol, reducing the concentration of detergent from 0,5; 0,25 and 0,1 % (w/v) respectively. The final stage of the BR purification involved the separation of the protein from low-molecular-weight impurities by gel-permeation chromatography on dextran Sephadex G-200 Column balanced with 0,09 M Tris-buffer (*pH* = 8,35) with 0,1 % (w/v) SDS-Na and 2,5 mM EDTA (output of the protein 8–10 mg).

The absorption spectrum of PM purified from carotenoids (4) and (5) (chromatographic purity 80–85 %) is shown in Figure 8 at various processing stages (*b*) and (*c*) relative to the native BR (*a*). Formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption spectrum of PM (Fig. 8*c*) – the main band with (1) with the absorption maximum at λ = 568 nm 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}; the additional low-intensity band with (2) at λ = 412 nm characterizes a minor impurity of a spectral form of *meta*-bacteriorhodopsin (M^{412}) (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwith (3) with λ = 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).

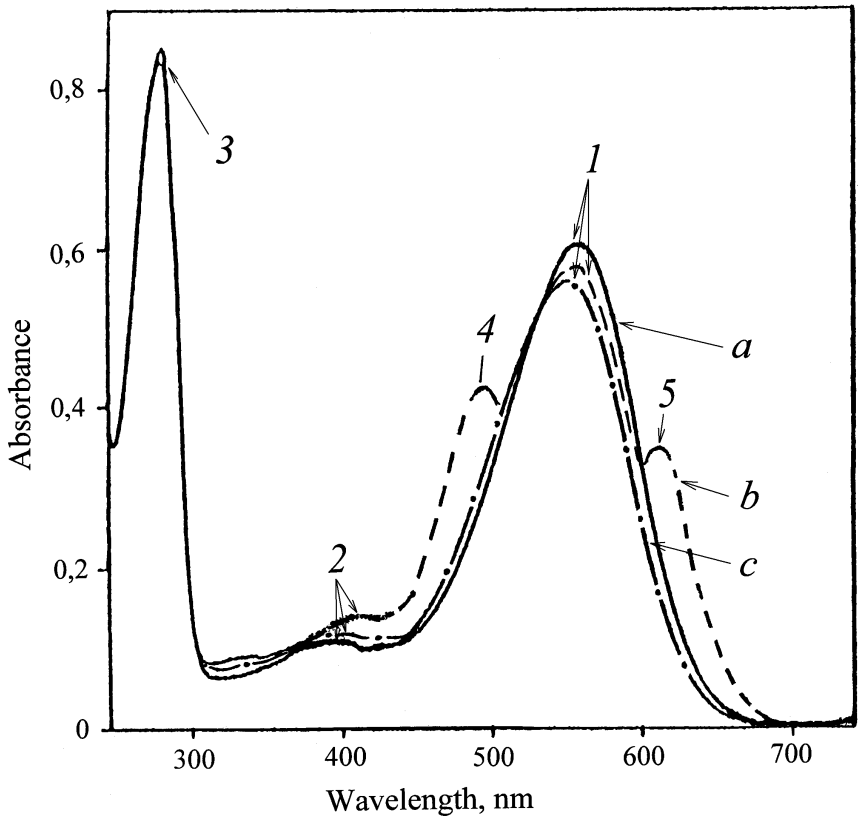

Fig. 8. The absorption spectra of the PM (50% (v/v) ethanol) at various stages of processing: (a) – natural BR; (b) – PM after intermediate treatment; (c) – PM purified from carotenoids. The bandwith (1) is the spectral form of BR^{568}, (2) – impurity of spectral form of meta-bacteriorhodopsin (M^{412}), (3) – the total absorption bandwith of aromatic amino acids, (4) and (5) – extraneous carotenoids. As a control used the native BR

The final stage of the BR purification involved the separation of the protein from low-molecular-weight impurities by gel-permeation chromatography (GPC). For this purpose the fractions containing the BR were 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. Elution was carried out at 20±25 ^{o}C with 1 mM Tris-HCl buffer (pH = 7,6) at 10 ml/cm^{2}·h. The data on purification of BR of phospholipids and carotenoids are shown in Table 1. 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 1: Summary results for the isolation and purification of BR by various methods

Sample	**PM content, mol PM/mol BR**	**Phospholipid and carotenoid removal, %**	**The 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.

The method for protein fractionation made it possible to obtain 8–10 mg of BR from 1 g of bacterial biomass. The homogeneity of BR was confirmed by electrophoresis in 12,5% (w/v) PAAG with 0,1% (w/v) SDS-Na and the regeneration of apomembranes with 13-*trans*-retinal.

3.4. Iodopsin

Iodopsin is a violet, light-sensitive pigment of the retinal cone cells, responsible for the light and color vision in mammals, the close analogue of rhodopsin. This pigment consists of a protein named photopsin linked with the chromophore, a retinal residue. According to the three-component theory of vision, it is believed that there has to be three types of this pigment and accordingly – three types of cones that are sensitive to blue, green and red light correspondingly. Evidently, there should be three types of cones – S, M, L types, each of which contains only their photosensitive pigment, corresponding to a specific opsin.

It is now established that this group of pigments comprises the isomorphic pigments, slightly different in composition and absorption spectra. Various opsins are different by amino acids in the molecule, and absorb light at several different wavelengths as do retinal-related molecules.

Iodopsin consists of three pigments – hlorolab, eritrolab and tsianolab. With the densitometry method W. Rushton studied the coefficient of light absorption in the photo layers of the retina with different wavelengths [31]. The hlorolab pigment absorbs the light rays corresponding to yellow-green (450–630 nm absorption band), the eritrolab – yellow and red (λ = 500-700 nm), and the third predicted pigment tsianolab – blue-green (λ = 500–700 nm) parts of the visible spectrum [32]. However, other different types of cones containing the only one pigment have not still yet been found.

The applying of intensive adapting yellow, purple and blue background, allowed get three different threshold curves in absorption spectra of iodopsin [33]. Making a correction for light absorption by the front media of the eye (lens and macular pigment yellow), G. Wald indicates as the maximums of the three pigments the peaks corresponding to the wavelengths: 430, 540 and 575 nm, which correspond to the photoreceptors cones of the retina that produce basic biosignals S, M, L (Table 2).

Table 2: The maximums of tsianolab, hlorolab and eritrolab pigments in absorption spectra and the sensitivity range (according to G. Wyszecki & Stiles, 1982)

Type of cones/photopigment (opsin)	Designation	Sensitivity range	Maximum sensitivity
S-cones/tsianolab (OPN1SW)	β	400–500 nm	420–440 nm
M-cones/hlorolab (OPN1MW)	γ	450–630 nm	534–545 nm
L-cones/eritrolab (OPN1LW)	ρ	500–700 nm	564–580 nm

It should be noted, however, that this interpretation does not fit correctly into the very basis of three-component hypothesis of color, as eritrolab and hlorolab have a sensitivity to the entire visible spectrum, and according to the three-component hypothesis they should be the narrow channeled, and their spectra sensitivity must comply strictly to the certain regions of the spectrum. In addition their maximums do not correspond to red and green colors (as postulated by the three-component hypothesis), their actual maxima correspond to eritrolab – yellow-red (orange) and hlorolab – yellow-green region of the spectrum.

3.5. The basic principles of the mechanism of color vision

It is established that the retina has three types of cone cells – S, M and L cells, having a different sensitivity to different parts of the visible range of the spectrum (Fig. 9). The cone cells of S type have a spectral range from 400 to 500 nm with a maximum peak at 420–440 nm, the cone cells of M type – from 450 to 630 nm with a maximum peak at 534–555 nm, while the cone cells of L type – from 500 nm to 700 nm with a maximum peak at 564–580 nm. As the curves of the sensitivity of the cone cells overlap, it is impossible for the monochromatic light to stimulate only one type of cone cells. The other types of cone cells react though to a lesser degree. The set of all possible values of the color combinations causing a visual reaction determines the human color space. The human brain generally can discern approximately 10 million of different colors.

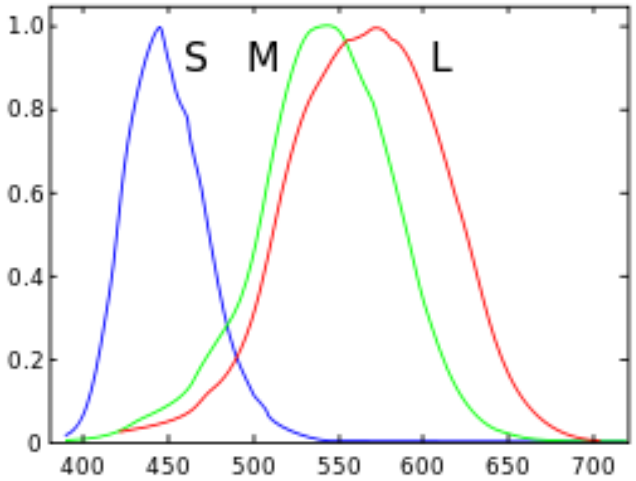

Fig. 9. The spectra of blue, green, and red rays in S, M and L points on the graphs of electromagnetic waves, which cones emit as the brightest ray signals rays of these points, from all the beams of monochromatic waves of substantive focused points with lengths in nm

The electromagnetic waves spectrum stimulates the different types of cone cells from the three types S, L and M to a different degree. Thus, the red light stimulates the L cone cells more than the M cone cells. On the contrary, the blue light stimulates the S cone cells in the strongest way. The yellow-green light provides a strong stimulation to the L and M cone cells, and a weaker stimulation to the S cone cells. The brain then combines the information from all types of cone cells for different wavelengths and analyzes them as different colors.

3.6. Studying the additive mixing of colors

The analysis of the activity of the three types of cones – S, L and M in the perception of different colors also shows how the brain "deciphers" the different colors. The foundation of this color analysis, shown in Figure 10, was made by M. Marinov and I. Ignatov in 2008. The maximum of the spectral sensitivity of the visual analyzer corresponds to the light green color corresponds to 560 nm in spectra (Fig. 10). However, it is not clear whether the green color perceived by brain is a combined mixing effect of yellow and blue colors, or whether it corresponds to a wavelength of the green color from the visible spectrum. The human brain can register the colors, i.e. the green color as a spectrometer, with certain lengths of the electromagnetic waves. It can also register the green color as a mixture of yellow and blue. However, the full perception of colors by the visual analyzer cannot be defined by a spectrometer.

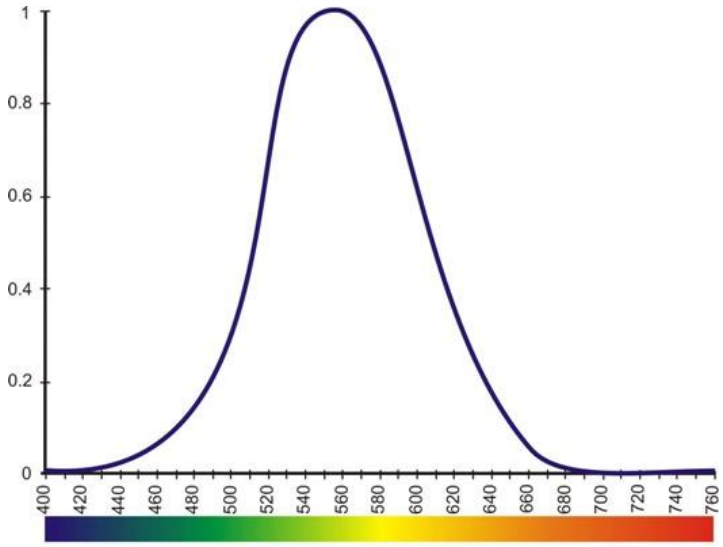

Fig. 10. The maximum of the spectral sensitivity of the visual analyzer on the graphs of the electromagnetic waves (M. Marinov & I. Ignatov, 2008)

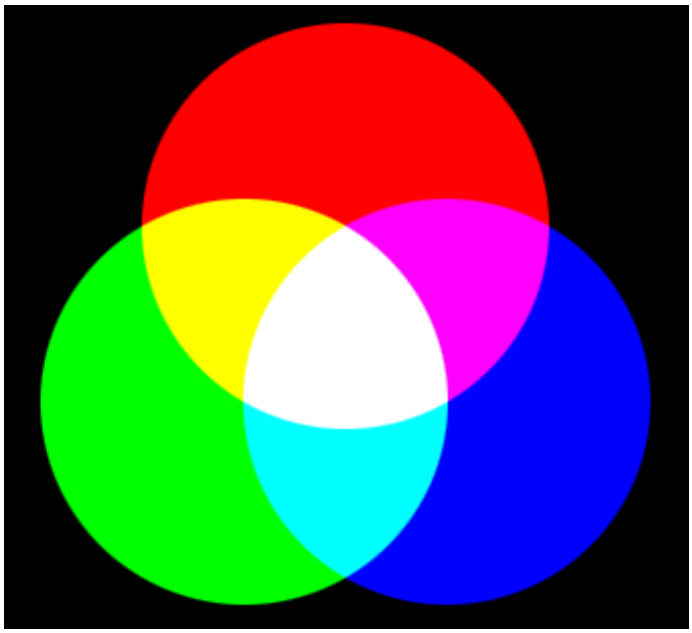

Fig. 11. Additive physical mixing of different colors by the visual analyzer (M. Marinov & I. Ignatov, 2008)

As an example via the mixing of electromagnetic waves that correspond to green and red color, the medium yellow color is obtained. In the mixing process of green and red, no medium color is obtained; the brain therefore perceives it as yellow color [34]. When there is an emission of electromagnetic waves that correspond to green and red color, the brain adopts an "average decision" – the yellow color (Fig. 11). Analogously, for the yellow and blue color, the brain adopts an "average decision" – the green color. This means that a spectral mixing of colors is observed between the blue-yellow and green-red pairs [35]. In its turn, green and blue color is perceived as cyan. The vision sensitivity furthermore is at its lowest for the violet, blue and red color. The mixing of electromagnetic waves that correspond to blue and red color is perceived as violet. In the mixing of electromagnetic waves that correspond to more colors, the brain does not perceive them as separate or average, but as a white color. Thus the notion of color is not determined solely by the wavelength. The analysis is performed by brain, and the notion of color is at its essence a product of our consciousness.

4. Conclusion

The mechanism of color perception by the visual analyzer has been carried out by us using photoreceptive chromo-protein rhodopsin as a basic model. A further research into the function of rhodopsin and other retina affiliated chromo-proteins as iodopsin will allow investigate in detail the mechanism of visual perception of light for better treatment of functional eye diseases in ophthalmology. It should be noted that rhodopsin up till now remains to be the most studied model chromoprotein of all GPCR-receptor family. This allowed us to carry out the comparative analysis and better analyze the functional properties of another analogous trans-membrane bacterial chromoprotein – bacteriorhodopsin isolated from purple membrane of halobacterium *H. halobium* in semi-preparative quantities, and study its structural-functional parameters and applications in bio-nanotechnologies.

Aknowlegements

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УДК 553.9 (470.22)

Новые нано- и биотехнологические аспекты применения бактериальных и животных фоторецепторных пигментов – бактериородопсина, родопсина и йодопсина

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Аннотация. В статье рассмотрены преимущественно структура и функции животных и бактериальных фоторецепторов пигментов (родопсин, йодопсин, бактериородопсин) и новые аспекты их нано- и биотехнологического применения. На примере бактериородопсина описан способ его выделения из пурпурных мембран аэробной экстремальной фотоорганотрофной галобактерии *Halobacterium halobium*, заключающийся в клеточном автолизе дистиллированной водой, обработкой бактериальной биомассы ультразвуком при 22 кГц, спиртовой экстракции низких и высокомолекулярных примесей, клеточной РНК, каротиноидов и липидов, коллоидном растворении в 0,5 % SDS-Na, фракционированием белка метанолом и гель-фильтрационную хроматографию на колонке Sephadex G- 200, уравновешенной 0,09 М трис-HCl буфером (*pH* = 6,76) с 0,1 % SDS-Na и 2,5 мМ ЭДТА. В рамках работы исследован механизма восприятия цвета зрительным аппаратом клетки, имеющим способность определять определенные диапазоны оптического спектра как цвета, а также был произведен анализ аддитивного смешения двух цветов. Показано, что при смешении электромагнитных волн с различными длинами волн, зрительный анализатор воспринимает их в виде отдельной или средней длины волны, соответствующей смешанному цвету.

Ключевые слова: зрение, родопсин, йодопсин, бактериородопсин, адитивное смешение цвета.