

# Halobacterium Halobacterium halobium in the Process of Biosynthesis of Deuterium-labeled Transmembrane Protein Bacteriorhodopsin

Oleg Mosin<sup>1</sup>

Ignat Ignatov<sup>2\*</sup>

1. Biotechnology Department, Moscow State University of Applied Biotechnology,

Talalikhina Street, 33, Moscow 109316, Russian Federation

2. Scientific Research Center of Medical Biophysics (SRCMB),

N. Kopernik Street, 32, Sofia 1111, Bulgaria

\* E-mail of the corresponding author: [mbioph@dir.bg](mailto:mbioph@dir.bg)

## Abstract

The semi-preparative biosynthesis of photochrome transmembrane protein bacteriorhodopsin (output 8–10 mg), labeled with deuterium on functionally important amino acid residues – [2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]phenylalanine, [3,5-<sup>2</sup>H<sub>2</sub>]tyrosine, and [2,4,5,6,7-<sup>2</sup>H<sub>5</sub>]tryptophan was carried out with using a photo-organotrophic halobacterium *Halobacterium halobium*. The protein was isolated from purple membranes 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, with the subsequent solubilization of final product with 0.5% (w/v) SDS-Na and fractionation by methanol. The homogeneity of the synthesized product, and the selectivity of deuterium incorporation into the molecule was proved by combination of preparative and analytical protein methods including electrophoresis in 12.5% (w/v) PAAG with 0.1% (w/v) SDS-Na, gel filtration chromatography on Sephadex G-200, and electron impact mass-spectrometry of methyl esters of N-Dns-[<sup>2</sup>H]derivatives of amino acids after their separation by reverse-phase HPLC.

**Keywords:** *Halobacterium halobium*, bacteriorhodopsin, [2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]Phe, [3,5-<sup>2</sup>H<sub>2</sub>]Tyr, [2,4,5,6,7-<sup>2</sup>H<sub>5</sub>]Trp, biosynthesis, EI mass-spectrometry, RP-HPLC.

## 1. Introduction

Bacteriorhodopsin (BR), named by analogy to the visual apparatus of mammalian protein rhodopsin, was isolated from the cell membrane of extreme photo-organotrophic halobacteria *Halobacterium halobium* in 1971 by D. Osterhelt and W. Stohenius (Oesterhelt & Stoeckenius, 1971). This photo-transforming transmembrane protein with the molecular weight ~26.5 kDa represents

chromoprotein determining the purple-red colour of halophilic bacteria, which contains as chromophore group an equimolar mixture of 13-*cis*- and 13-*trans*-retinol C20 carotenoid, bound by Schiff base (as in the visual animal pigments) with Lys-216 residue of the protein (Oesterhelt, 1988). Owing to its structure, 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. This mechanism is called “non-chlorophyll photosynthesis”, in contrast to the plant photosynthesis implemented with the participation of chlorophyll. In this mechanism, at absorption of a light photon 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 (Haupt *et al.*, 1997). As a result, between the internal and external surface of the membrane forms a concentration gradient of  $H^+$ , that leads that illuminated halobacteria 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, allowing halobacteria develop in the dark via switching of the photosynthetic metabolism on heterotrophic metabolism. Thus, 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 for BR are dynamic and vary depending on the method for preparation of purple membranes (PM), the polymer matrix and its composition.

BR is the focus of bio- and nanotechnology mainly 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 (Hampp & Oesterhelt, 2004; Vought & Birge, 1999; Gui-Ying *et al.*, 2012). Additionally, BR is very attractive as a model for studies of functional activity and structural properties of photo-transforming cell membrane proteins in the native and photo-converting membranes (Ignatov & Mosin, 2014). For these studies it is useful to enter into the protein molecule a deuterium label ( $^2H$ ), which allows to apply for evaluating of the structure by NMR-method (LeMaster, 1990). In this aspect great scientific and practical interest has BR labeled with deuterium on the residues of functionally important aromatic amino acids – phenylalanine, tyrosine and tryptophan involved in the hydrophobic interaction of the polypeptide chain of the protein with the lipid bilayer of the cell membrane.  $^2H$ -labeled aromatic amino acids can be synthesized in gram scale quantities by isotopic exchange ( $H-^2H$ ) in molecules of the protonated amino acids; for analyzing deuterium enrichment levels the EI mass-spectrometry of N-5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl) amino acid derivatives may be used (Mosin *et al.*, 1994; Mosin *et al.*, 1996; Mosin *et al.*, 1999).

The purpose of this research was to study the micro preparative biosynthesis of BR labeled with deuterium on the residues of functionally important aromatic amino acids –  $[2,3,4,5,6-^2H_5]$ phenylalanine,  $[3,5-^2H_2]$ tyrosine and  $[2,4,5,6,7-^2H_5]$ tryptophan, for the reconstruction of artificial membranes, as well as to study the levels of deuterium enrichment of the BR molecule by EI mass spectrometry in combination with RP HPLC.

## 2. Materials and methods

### 2.1. Bacterial strain

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 agarose media with 2% (w/v) peptone and 4.3 M NaCl.

## 2.2. Chemicals

For the synthesis of aromatic [<sup>2</sup>H]amino acids were used the protonated *D,L*-amino acids (“Reanal”, Hungary), <sup>2</sup>H<sub>2</sub>O (99.9 atom.% <sup>2</sup>H), <sup>2</sup>HCl (95.5 atom.% <sup>2</sup>H), and <sup>2</sup>H<sub>2</sub>SO<sub>4</sub> (97.5 atom.% <sup>2</sup>H), purchased from the Russian Research Center “Isotope” (St. Petersburg, Russia). For the synthesis of methyl esters of DNS-amino acids used 5-(dimethylamino) naphthalene-1-sulfonyl chloride (Dns-chloride) (“Sigma”, USA) and diazomethane, prepared from N-nitrosourea (“Merck”, Germany). The inorganic salts were crystallized in <sup>2</sup>H<sub>2</sub>O, <sup>2</sup>H<sub>2</sub>O was distilled over KMnO<sub>4</sub> with subsequent control of the isotope purity by <sup>1</sup>H-NMR spectroscopy on a Bruker WM-250 (“Bruker Daltonics” Germany) with a working frequency 70 MHz (internal standard Me<sub>4</sub>Si).

## 2.3. Synthesis of L-[2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]phenylalanine

40 g of phenylalanine was dissolved in 300 ml of 85% (v/v) <sup>2</sup>H<sub>2</sub>SO<sub>4</sub> (in <sup>2</sup>H<sub>2</sub>O) and heated with water reflux condenser at 50–60 °C under stirring for 3 days. Upon completion the reaction mixture was cooled, neutralized with 30% (v/v) NH<sub>4</sub>OH to pH = 5.5. The product was extracted with ethanol. Yield 24 g (58.7%), *T<sub>m</sub>* = 271–273 °C; [ $\alpha$ ]<sub>d</sub><sup>25</sup> = 4.47 (5 M HCl); *pK<sub>a</sub>* = 2.20 (COOH), 9.31 (NH<sub>2</sub>); *R<sub>f</sub>* = 0.6 (A). UV-spectrum (0.1 M HCl):  $\lambda_{\max}$  nm [( $\epsilon$  M<sup>-1</sup>cm<sup>-1</sup>): 257.5 [( $\epsilon$  195)]. <sup>1</sup>H-NMR spectrum (<sup>2</sup>H<sub>2</sub>SO<sub>4</sub> in <sup>2</sup>H<sub>2</sub>O) ( $\delta$ , ppm):  $\delta$  3.25 (2H, H $\beta$ ),  $\delta$  4.4 (1H, H $\alpha$ ),  $\delta$  7.2–7.4 (0.07H), 95 atom.% <sup>2</sup>H. EI mass spectrum [ $M$ ]<sup>+</sup> *m/z* (*I*, %): 165 (34), methyl ester of N-Dns-[2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]Phe: 417 (14), 418 (6).

## 2.4. Synthesis of L-[3,5-<sup>2</sup>H<sub>2</sub>]tyrosine

100 g of tyrosine was dissolved in 150 ml of 3 M <sup>2</sup>H<sub>2</sub>SO<sub>4</sub>. The reaction mixture was heated for 2 days at 40–50 °C with water reflux condenser under a slow stream of dry nitrogen. The solution was neutralized with 30% (v/v) NH<sub>4</sub>OH to pH = 4.5 and cooled for 24 h at 4 °C. The crystalline product was filtered, washed with <sup>2</sup>H<sub>2</sub>O and dried at 10 mm Hg. Yield 90 g (86.5%); *T<sub>m</sub>* = 316–317 °C; [ $\alpha$ ]<sub>d</sub><sup>25</sup> = 10.03 (5 M HCl); *pK<sub>a</sub>* = 2.20 (COOH), 9.21 (NH<sub>2</sub>); *R<sub>f</sub>* = 0.45 (A). UV-spectrum (0.1 M HCl)  $\lambda_{\max}$  nm [( $\epsilon$  M<sup>-1</sup>cm<sup>-1</sup>): 223 [( $\epsilon$  8200), 274.5 ( $\epsilon$  1340)]. <sup>1</sup>H-NMR spectrum (1M <sup>2</sup>HCl) ( $\delta$ , ppm):  $\delta$  3.32 (2H),  $\delta$  4.35 (1H),  $\delta$  6.9 (1H),  $\delta$  7.2 (2H), 96 atom.% <sup>2</sup>H. EI mass spectrum [ $M$ ]<sup>+</sup> *m/z* (*I*, %): 181 (21), methyl ester of N-Dns-[3,5-<sup>2</sup>H<sub>2</sub>]Tyr: 429 (15), 430 (5).

## 2.5. Synthesis of L-[2,4,5,6,7-<sup>2</sup>H<sub>5</sub>]tryptophan

100 ml of 40% (v/v) <sup>2</sup>H<sub>2</sub>O was placed in a round bottom flask and cooled in an ice bath. 80 ml of trifluoroacetic anhydride (0.5 mol) was added dropwise with stirring. The reaction mixture was kept for 2 h at 4 °C, then 25 g of tryptophan was added portionwise. The reaction mixture was kept for 3 days in the dark at 22 °C, the solvent was removed at 10 mm Hg., neutralized with 30% (v/v) NH<sub>4</sub>OH to pH = 5.9, and cooled for 1 day at 4 °C. The crystalline product was filtered, washed with <sup>2</sup>H<sub>2</sub>O and dried at 10 mm Hg. Yield 19 g (60.3%); *T<sub>m</sub>* = 283–285 °C; [ $\alpha$ ]<sub>d</sub><sup>25</sup> = 2.8 (1 M HCl); *pK<sub>a</sub>* =

2.46 (COOH), 9.41 (NH<sub>2</sub>);  $R_f = 0.5$  (A). UV-spectrum (0.1 M HCl)  $\lambda_{\max}$  nm [ $\epsilon$  M<sup>-1</sup>cm<sup>-1</sup>]: 218 [ $\epsilon$  3350), 278 ( $\epsilon$  5550), 287.5 ( $\epsilon$  4550)]. <sup>1</sup>H-NMR spectrum (CF<sub>3</sub>COOH in <sup>2</sup>H<sub>2</sub>O) ( $\delta$ , ppm):  $\delta$  3.4 (2H, H $\beta$ ),  $\delta$  4.4 (1H, H $\alpha$ ),  $\delta$  7.3 (1H, H $\epsilon$ ),  $\delta$  7.2–7.4 (0.1H, In-H), 98 atom.% <sup>2</sup>H. EI mass spectrum [M]<sup>+</sup>  $m/z$  (I, %): 204(28), methyl ester of N-Dns-[2,4,5,6,7-<sup>2</sup>H<sub>5</sub>]Trp: 455(9), 456(11).

### 2.6. Synthesis of N-Dns-[<sup>2</sup>H]amino acids

To 4 mg of the dry hydrolyzate BR 1 ml of 2 M NaHCO<sub>3</sub> (pH = 9–10) was added portionwise with stirring 25.6 mg of Dns-chloride in 2 ml of acetone. The reaction mixture was kept for 1 h under stirring at 40 °C, acidified with 2 N HCl to pH = 3.0 and extracted with ethyl acetate (3 times  $\times$  5 ml). The combined extract was washed with distilled <sup>2</sup>H<sub>2</sub>O to pH = 7.0 (2 times  $\times$  2 ml), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed at 10 mm Hg. Yield 5.3 mg (78%).

### 2.7. Synthesis of methyl esters of N-Dns-[<sup>2</sup>H]amino acids

3 g. of wet N-nitroso-N-methylurea was added to 20 ml of 40% (v/v) KOH in 40 ml of diethyl ether and then stirred on a water bath with ice for 15–20 min for obtaining diazomethane. After the completion of gas release, the ether layer was separated, washed with distilled water to pH = 7.0, dried with anhydrous Na<sub>2</sub>SO<sub>4</sub>, and used for the treatment of N-Dns-amino acids. Yield 17.4 mg (69%).

### 2.8. Biosynthesis of BR

Biosynthetic BR (yield 8–10 mg from 1 g biomass) was obtained in synthetic (SM) medium in which the protonated phenylalanine, tyrosine and tryptophan were replaced by their deuterated analogs – [2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]phenylalanine, [3,5-<sup>2</sup>H<sub>2</sub>]tyrosine, and [2,4,5,6,7-<sup>2</sup>H]tryptophan (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-lysine – 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<sub>4</sub>·7H<sub>2</sub>O – 20; KCl – 2; NH<sub>4</sub>Cl – 0.5; KNO<sub>3</sub> – 0.1; KH<sub>2</sub>PO<sub>4</sub> – 0.05; K<sub>2</sub>HPO<sub>4</sub> – 0.05; Na<sup>+</sup>-citrate – 0.5; MnSO<sub>4</sub>·2H<sub>2</sub>O – 3 · 10<sup>-4</sup>; CaCl<sub>2</sub>·6H<sub>2</sub>O – 0.065; ZnSO<sub>4</sub>·7H<sub>2</sub>O – 4 · 10<sup>-5</sup>; FeSO<sub>4</sub>·7H<sub>2</sub>O – 5 · 10<sup>-4</sup>; CuSO<sub>4</sub>·5H<sub>2</sub>O – 5 · 10<sup>-5</sup>; glycerol – 1.0, biotin – 1 · 10<sup>-4</sup>; folic acid – 1.5 · 10<sup>-4</sup>, vitamin B<sub>12</sub> – 2 · 10<sup>-5</sup>. 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 a shaker (“Birad Labs”, Hungary) under intense aeration and monochromatic illumination (3 lamps  $\times$  1.5 lx). All further manipulations with BR isolation were carried out with the use of a photomask lamp equipped with an orange light filter.

### 2.9. 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<sub>2</sub>O 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<sub>2</sub>O and disintegrated by infrasound sonication (22 kHz, 3 times  $\times$  5 min) in an ice bath (0 °C). The cell homogenate after washing with dist. H<sub>2</sub>O was resuspended in 10 ml of buffer containing

125 mM NaCl, 20 mM MgCl<sub>2</sub>, 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  $D_{280}/D_{568}$  ( $\epsilon_{280} = 1.1 \cdot 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ;  $\epsilon_{568} = 6.3 \cdot 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) (Neugebauer *et al.*, 1978; Mosin *et al.*, 2012). PM regeneration is performed as described in (Rudiger *et al.*, 1997; Mosin *et al.*, 2013). Yield of PM fraction 120 mg (80–85%).

### **2.10. Isolation of BR**

Fraction PM (in H<sub>2</sub>O) (1 mg/ml) was dissolved in 1 ml of 0.5% (w/v) 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% (w/v). Crystal protein (output 8–10 mg) was washed with cold distilled. <sup>2</sup>H<sub>2</sub>O (2 times × 1 ml) and centrifuged (1200 g, 15 min).

### **2.11. 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 (“Pharmacia”, 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 EDTA. Elution proceeded by 0.09 M Tris-borate buffer containing 0.5 M NaCl, pH = 8.35 at a flow rate 10 ml/cm<sup>2</sup>·h. Combined protein fraction was subjected to freeze-drying, in sealed glass ampoules (10 × 50 mm) and stored in frost camera at -10 °C.

### **2.12. Electrophoresis of BR**

The procedure was performed in 12.5% (w/v) polyacrylamide gel (PAAG) containing 0.1% (w/v) SDS. 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 level.

### **2.13. Hydrolysis of BR**

4 g of dry protein was placed into glass ampoules (10 × 50 mm), and 5 ml 4 N Ba(OH)<sub>2</sub> was added. The mixture was kept at 110 °C for 24 h. The reaction mixture was suspended with 5 ml of hot distilled water and neutralized with 2 N H<sub>2</sub>SO<sub>4</sub> to pH = 7.0. The precipitate of BaSO<sub>4</sub> was removed by centrifugation at 200 g for 10 min, and the supernatant was evaporated in a rotor evaporator at 40 °C.

### **2.14. RP-HPLC**

RP-HPLC was carried out on a liquid chromatograph Knauer Smartline (“Knauer”, Germany)

equipped with a pump Gilson (“Gilson Inc.”, USA), UF-2563 detector and integrator CR-3A (“Shimadzu”, Japan),  $t = 20 \pm 25$  °C, using a column (250×10 mm) with a stationary reverse phase Separon C18 (“Kova”, Slovakia), eluent: (A) – acetonitrile-trifluoroacetic acid 100:0,1–0,5% (v/v) and (B) – acetonitrile–100% (v/v). The results on gradient elution are shown in Table 1. The yield of individual methyl esters of N-Dns- $^{2}\text{H}$  amino acids – 75–89%; chromatographic purity – 95–98%.

Table 1. The results of one-step gradient separation of a mixture of methyl esters of N-Dns  $^{2}\text{H}$  amino acids from the hydrolyzate of the BR by RP HPLC on a column 250×10 mm,  $t = 20 \pm 25$  °C, with octadecylsilane silica gel Separon SGX C 18,7  $\mu\text{m}$

Number processing	Components of the mobile phase,% vol.		Elution time, min
	A*	B**	
1	90	10	10
2	80	20	10
3	60	40	10
5	50	50	10
6	30	60	5
8	20	80	5
9	10	90	5
10	0	100	5

Notes: \* A – acetonitrile-trifluoroacetic acid 100 : 0.1–0.5% (v/v);

\*\* B – acetonitrile–100% (v/v)

### 2.15. EI mass spectrometry

Mass spectra of methyl esters of N-Dns-amino acid derivatives were obtained by electron impact on MB-80A device (“Hitachi”, Japan) at energy of ionizing electrons 70 eV, accelerating voltage of 8 kV and the cathode temperature 180–200 °C. Scanning was carried out at a resolution of 7500 relative units using 10% sharpness.

## 3. Results and discussion

### 3.1. BR biosynthesis

The strategy for biosynthesis of  $^{2}\text{H}$ -labeled BR using a strain of extreme photo-organotrophic halobacterium *Halobacterium halobium* was determined by the study of the principal possibility for obtaining  $^{2}\text{H}$ -labeled photochrome transmembrane proteins in semi-micropreparative quantities for reconstruction of artificial membranes in  $^{2}\text{H}_2\text{O}$  and BR-containing nanofilms. [2,3,4,5,6- $^{2}\text{H}_5$ ]phenylalanine, [3,5- $^{2}\text{H}_2$ ]tyrosine, and [2,4,5,6,7- $^{2}\text{H}_5$ ]tryptophan play important role in hydrophobic interacting of BR molecule with the lipid bilayer of the cell membrane. They are resistant to the isotopic exchange ( $\text{H}$ – $^{2}\text{H}$ ) reactions in aqueous solutions under growth conditions and may be easily detected by IE spectrometry after the derivatization to the methyl esters of

N-Dns-amino acids.  $^2\text{H}$ -labeled BR was obtained via growth of halobacterium *H. halobium* on synthetic medium (4.3 M NaCl) with [2,3,4,5,6- $^2\text{H}_5$ ]phenylalanine, [3,5- $^2\text{H}_2$ ]tyrosine and [2,4,5,6,7- $^2\text{H}_5$ ]tryptophan. Under optimal growing conditions (incubation period 4–5 days, temperature 35 °C, illumination with monochromatic light at  $\lambda = 560$  nm) in cells were synthesized the purple carotenoid pigment, the spectral ratio of protein and chromophore molecule fragments  $D_{280}/D_{568} = 1.5: 1.0$  in which was identical to the natural BR. The growth of the halobacterium on a synthetic medium (Fig. 1b) was practically as the same as in the control (Fig. 1a) on the protonated growth medium with protonated amino acids, that significantly simplifies the optimization of conditions for the biosynthesis of  $^2\text{H}$ -labeled BR, which consists in the equivalent replacing of protonated aromatic amino by their deuterated analogues – [2,3,4,5,6- $^2\text{H}_5$ ]phenylalanine (0.26 g/l), [3,5- $^2\text{H}_2$ ]tyrosine (0.2 g/l) and [2,4,5,6,7- $^2\text{H}_5$ ]tryptophan (0.5 g/l).

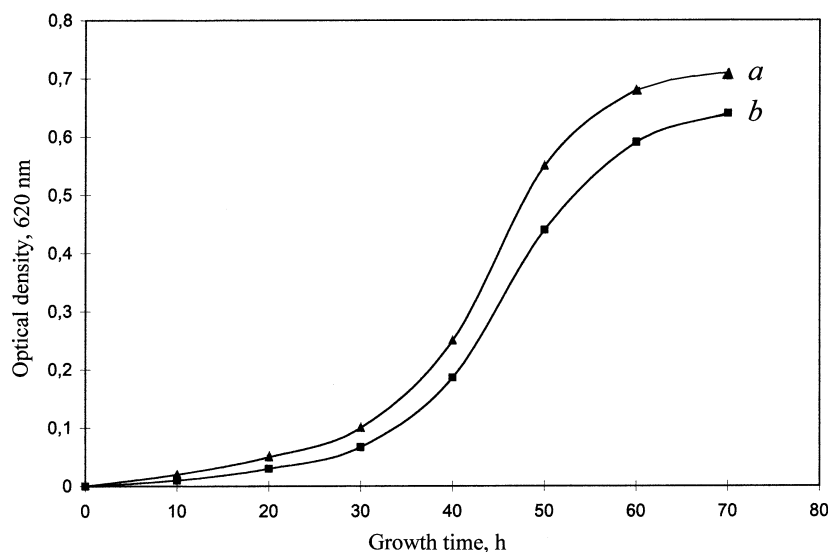


Figure 1. Growth dynamics of *H. halobium* under various experimental conditions: a) – protonated synthetic medium; b) – synthetic medium with [2,3,4,5,6- $^2\text{H}_5$ ]Phe (0.26 g/l), [3,5- $^2\text{H}_2$ ]Tyr (0.2 g/l) and [2,4,5,6,7- $^2\text{H}_5$ ]Trp (0.5 g/l). The incubation period: 4–5 days, temperature: 35 °C, illumination under monochrome light at  $\lambda = 560$  nm.

The main stages of the experiment were: growing of a strain-producer *H. halobium* on synthetic medium with [2,3,4,5,6- $^2\text{H}_5$ ] phenylalanine (0.26 g/l), [3,5- $^2\text{H}_2$ ] tyrosine (0.2 g/l) and [2,4,5,6,7- $^2\text{H}_5$ ] tryptophan (0.5 g/l), the separation of cell content, isolation of purple membrane (PM) fraction, removal of low-and high-molecular impurities, cellular RNA, pigments (preferably carotenoids) and lipids, fractionation of solubilized in 0.5% (w/v) SDS-Na protein by methanol, purification on Sephadex G-200, and final electrophoresis 12.5% (w/v) PAAG with 0.1% (w/v) SDS-Na. Because 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. Fraction PM 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 gel permeation column chromatography on Sephadex G-200. Removing of carotenoids, consisting in repeated treatment of PM with 50% (v/v) EtOH ethanol at 4 °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) (degree of chromatographic purity of 80–85%), as shown in Figure 2 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. 2*c*) – the main bandwidth (1) with the absorption maximum at  $\lambda = 568$  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<sub>568</sub>; additional low-intensity bandwidth (2) at  $\lambda = 412$  nm characterizes a minor impurity of a spectral form of meta-bacteriorhodopsin M<sub>412</sub> (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwidth (3) with  $\lambda = 280$  nm is determined by the absorption of aromatic amino acids in the polypeptide chain of the protein (for native BR  $D_{280}/D_{568} = 1.5 : 1.0$ ).

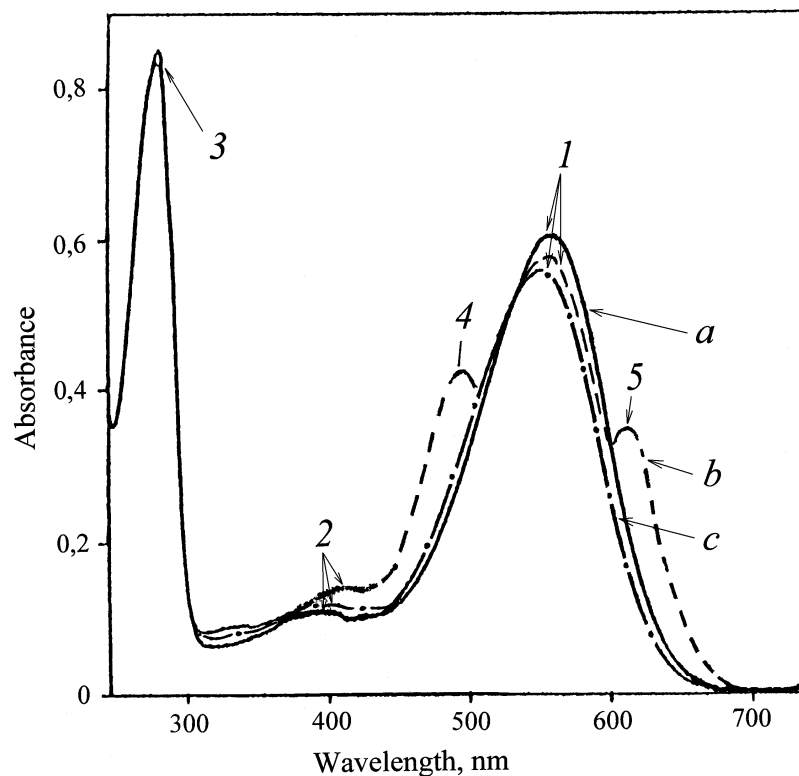


Figure 2. The absorption spectra of the PM (50% (v/v) EtOH) at various stages of processing: (*a*) – natural BR; (*b*) – PM after intermediate treatment; (*c*) – PM purified from carotenoids. The bandwidth (1) is the spectral form of BR<sub>568</sub>, (2) – impurity of spectral form of meta-bacteriorhodopsin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



### 3.2. Isolation and 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 bilipid 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 of 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  $\alpha$ -helical configuration (Nonella *et al.*, 1991). Therefore, there is no need the use organic solvents as acetone, methanol and chloroform for purification of lipids and protein, and precipitation and delipidization are 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 solubilized in a 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 BR purification involved the separation of the protein from low-molecular-weight impurities by gel-permeation chromatography (GPC). For this purpose the fractions containing 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 °C with 1 mM Tris-HCl buffer (pH = 7,6) at 10 ml/cm<sup>2</sup>·h. The data on purification of BR of phospholipids and carotenoids are shown in Table 2. 84% of phospholipids was removed by five washes (65, 70 and 76% was removed by 1st, 2nd and 3rd wash respectively). The total endogenous phospholipid 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.

The method for protein fractionation made it possible to obtain 8–10 mg of  $^2\text{H}$ -labeled BR from 1 g of bacterial biomass. The homogeneity of BR satisfy the requirements for reconstruction of native membranes, and was confirmed by electrophoresis in 12,5% (w/v) PAAG with 0,1% (w/v) SDS-Na, regeneration of apomembranes with 13-*trans*-retinal, and RP-HPLC of methyl esters of N-DNS- $^2\text{H}$ ]amino acids. Micropreparative output of BR was no barrier to further mass spectrometric analysis. However, it must be emphasized that to ensure high yields of protein it needs to accumulate more row biomass feedstock.

### 3.3. Hydrolysis of BR

Conditions of hydrolysis of  $^2\text{H}$ -labeled protein were determined to prevent the isotopic ( $\text{H}-^2\text{H}$ ) exchange of hydrogen by deuterium in the molecules of aromatic amino acids, as well as to retain tryptophan in the protein hydrolysate. We considered two alternatives variants - acid and alkaline hydrolysis. Acid hydrolysis of the protein in standard conditions (6 N HCl or 8 N  $\text{H}_2\text{SO}_4$ , 110 °C, 24 h) is known to induce complete degradation of tryptophan and partial degradation of serine, threonine, and several other amino acids in the protein (Zvonkova *et al.*, 1970), which do not play a significant role for this study. Modification of this method consists in adding to the reaction mixture of phenol, thioglycolic acid,  $\beta$ -mercaptoethanol, can save up to 80–85% tryptophan (Penke *et al.*, 1974). Using of p-toluenesulfonic acid with 0.2% (v/v) 3-(2-aminoethyl) indole or 3 M mercapto sulfoacid is also effective to maintain a tryptophan (up to 93%) (Liu & Chang, 1971). However, these methods are possess an essential disadvantage, because during acid hydrolysis at high speed occurs isotopic ( $\text{H}-^2\text{H}$ ) exchange of aromatic protons(deuterons) in molecules of tryptophan, tyrosine and histidine as well as protons at C3 position of aspartic and C4 glutamic acid (Pshenichnikova *et al.*, 1995). Therefore, even carrying out of hydrolysis in deuterated reagents (6N  $^2\text{HCl}$ , 4N  $^2\text{H}_2\text{SO}_4$  in  $^2\text{H}_2\text{O}$ ) does not derive the real data about the inclusion of the deuterium into the protein.

Under conditions of alkaline hydrolysis (4N  $\text{Ba}(\text{OH})_2$  or 4N NaOH, 110 °C, 24 hours) the reactions of isotopic ( $\text{H}-^2\text{H}$ ) exchange were almost not occurred (except for a proton (deuteron) at C2 atom of histidine, and tryptophan not destroyed. These factors determined the choice of this method of hydrolysis in our research. Simplification of the procedures for the allocation of amino acid mixture from protein hydrolyzate due to the neutralization of  $\text{H}_2\text{SO}_4$  (in  $^2\text{H}_2\text{O}$ ) was the reason for choosing as hydrolysing agent 4N  $\text{Ba}(\text{OH})_2$ . Possible *D,L*-amino acid racemization by alkaline hydrolysis did not affect the result of the subsequent mass spectrometric study of the level of deuteration of amino acid molecules.

### 3.4. Studying of the deuterium labeling of BR

To study the deuterium labelling of BR molecule EI mass spectrometry was used after modification of amino acid mixture of the hydrolyzate into methyl esters of N-Dns- $^2\text{H}$ ]amino acids. To obtain reproducible results on the deuteration of  $^2\text{H}$ -labeled protein first was recorded a total scan EI mass

spectrum of the mixture of methyl esters of N-Dns- $^{2}\text{H}$  amino acids derived from the BR hydrolyzate. The level of deuteration was calculated from the peak of the molecular ion  $[\text{M}]^{+}$  of amino acid derivatives relative to the control. Then, the separation of methyl esters of N-Dns- $^{2}\text{H}$  aromatic amino acids was performed by RP HPLC to record EI mass spectra for each individual amino acid derivat. EI mass spectrum of the mixture of methyl esters of N-Dns- $^{2}\text{H}$  amino acids as shown in Figure 3 (scanning at  $m/z$  50–640, base peak at  $m/z$  527, 100%), is characterized by continuity: the peaks in the range at  $m/z$  50–400 on the scale of the mass numbers are fragments of metastable ions, low molecular weight impurities, as well as products of chemically modified amino acids. Analyzed  $^{2}\text{H}$  aromatic amino acids occupied scale mass numbers at  $m/z$  415–456, are mixtures of molecules containing various numbers of deuterium atoms. Therefore, the molecular ions  $[\text{M}]^{+}$  were polymorphously split into individual clusters displaying a statistical set of  $m/z$  values depending on a number of hydrogen atoms in the molecule. Taking into account the effect of isotopic polymorphism, the level of deuterium enrichment in  $^{2}\text{H}$  amino acid molecules was determined using the most commonly encountered peak of the molecular ion  $[\text{M}]^{+}$  in each cluster with mathematically averaged value of  $[\text{M}]^{+}$  (Fig. 3). Thus, for phenylalanine molecular ion peak was determined by  $[\text{M}]^{+}$  at  $m/z$  417, 14% (instead of the  $[\text{M}]^{+}$  at  $m/z$  412, 20% for non-labeled derivative (unlabeled peaks of amino acid derivatives are not shown)), tyrosine –  $[\text{M}]^{+}$  at  $m/z$  429, 15% (instead of  $[\text{M}]^{+}$  at  $m/z$  428, 13%), tryptophan –  $[\text{M}]^{+}$  at  $m/z$  456, 11% (instead of  $[\text{M}]^{+}$  at  $m/z$  451, 17%). The level of deuterium enrichment corresponding to the increase of molecular weight was for  $^{2}\text{H}$  tyrosine 1 (90 atom.%  $^{2}\text{H}$ ),  $^{2}\text{H}$  phenylalanine – 5 (95 atom.%  $^{2}\text{H}$ ) and  $^{2}\text{H}$  tryptophan – 5 (98 atom.%  $^{2}\text{H}$ ) deuterium atoms. This result coincides with the data on the initial level of deuterium enrichment of aromatic amino acids –  $[\text{3,5-}^{2}\text{H}_2]\text{Tyr}$ ,  $[\text{2,3,4,5,6-}^{2}\text{H}_5]\text{Phe}$  and  $[\text{2,4,5,6,7-}^{2}\text{H}_5]\text{Trp}$ , added to the growth medium and indicates a high selectivity of inclusion of aromatic  $^{2}\text{H}$  amino acids into the BR molecule. Deuterium was detected in all residues of aromatic amino acids (Table). However, the presence in the EI mass spectrum peaks  $[\text{M}]^{+}$  of protonated and semi-deuterated phenylalanine analogues with  $[\text{M}]^{+}$  at  $m/z$  413–418, tyrosine – with  $[\text{M}]^{+}$  at  $m/z$  428–430 and tryptophan – with  $[\text{M}]^{+}$  at  $m/z$  453–457 with different levels of contributions to the deuterium enrichment of molecules testifies about conservation of the minor pathways of biosynthesis of aromatic amino acids *de novo*, resulting in the dilution of the deuterium label in molecules, that evidently is determined by the conditions of biosynthesis of  $^{2}\text{H}$ -labeled BR (Table).

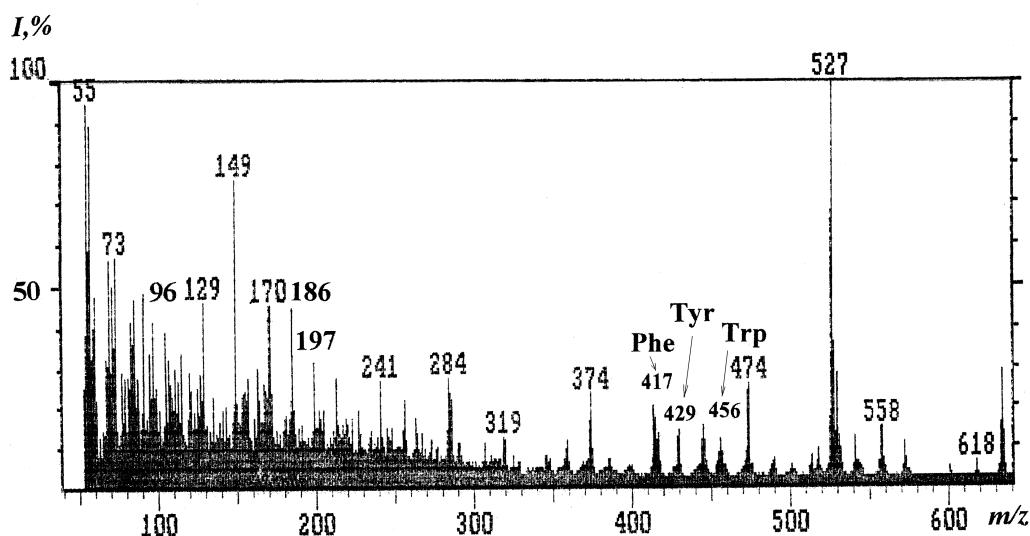


Figure 3. Full scan EI mass spectrum of methyl ester of N-Dns- $^{2}\text{H}$  derivatives of aromatic amino acids isolated from the BR hydrolyzate, obtained on synthetic medium with  $[2,3,4,5,6\text{-}^{2}\text{H}_5]\text{Phe}$  (0.26 g/l),  $[3,5\text{-}^{2}\text{H}_2]\text{Tyr}$  (0.2 g/l) and  $[2,4,5,6,7\text{-}^{2}\text{H}_5]\text{Trp}$  (0.5 g/l) (energy: 70 eV, accelerating voltage: 8 kV, temperature: 180–200 °C). Hydrolysis conditions: 4 N  $\text{Ba}(\text{OH})_2$  (in  $^{2}\text{H}_2\text{O}$ ), 110 °C, 24 h. Molecular ion peaks represented by the symbols of amino acids correspond to their derivatives;  $I$  – relative intensity (%).

According to the mass spectrometric analysis the molecular ion peaks  $[\text{M}]^+$  of methyl esters of N-Dns- $^{2}\text{H}$  derivatives of aromatic  $^{2}\text{H}$  amino acids have a low intensity in EI mass spectra and were polymorphously split, so the areas of of the molecular enrichment were strongly broadened. Moreover, mass spectra of the mixture components are additive, so the mixture can be analyzed only if the spectra of the various components are recorded under the same conditions. These calculations provide for the solution of the system of  $n$  equations in  $n$  unknowns for a mixture of  $n$  components. For components, the concentration of which exceeds 10 mol.%, the accuracy and reproducibility of the analysis makes up  $\pm 0.5$  mol.% (at 90% confidence probability). Therefore, to obtain reproducible results on deuteration levels it was necessary to chromatographically isolate individual derivatives of  $^{2}\text{H}$  amino acids from the protein hydrolyzate. For this aim was used RP-HPLC on on octadecylsilane silica gel Silasorb C18, the effectiveness of which was confirmed earlier by separation of a mixture of methyl esters of N-Dns- $^{2}\text{H}$  amino acids isolated from other microbial objects as methylotrophic bacteria and microalgae (Mosin & Ignatov, 2012; Mosin & Ignatov, 2013).

Table 3. The values of the molecular ion peaks  $[\text{M}]^+$  in the EI mass spectrum of methyl esters of N-Dns- $[2,3,4,5,6\text{-}^{2}\text{H}_5]\text{Phe}$ , N-Dns- $[3,5\text{-}^{2}\text{H}_2]\text{Tyr}$  and N-Dns- $[2,4,5,6,7\text{-}^{2}\text{H}_5]\text{Trp}$  and levels of their deuterium enrichment.

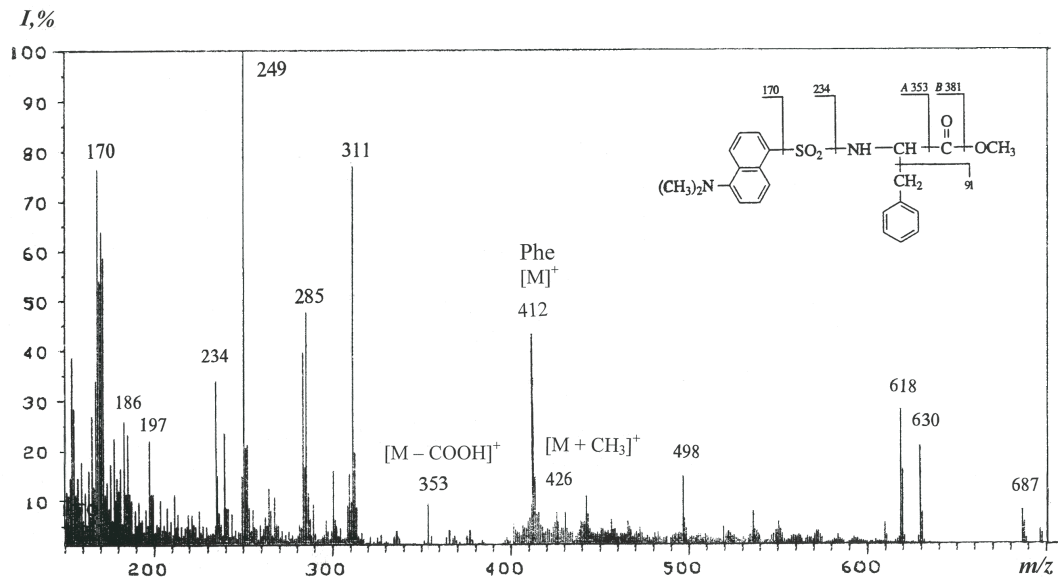
Compound	Value of $[\text{M}]^+$	Intensity, %	The total number of	Level of deuterium enrichment, % of the

			hydrogen atoms*	total number of hydrogen atoms **
N-Dns-[2,3,4,5,6- <sup>2</sup> H <sub>5</sub> ]Phe-OMe	413	7	1	13
	414	18	2	25
	415	15	3	38
	416	11	4	50
	417	14	5	63
	418	6	6	75
N-Dns-[3,5- <sup>2</sup> H <sub>2</sub> ]Tyr-OMe	428	12	–	–
	429	15	1	14
	430	5	2	29
N-Dns-[2,4,5,6,7- <sup>2</sup> H <sub>5</sub> ]Trp-OMe	453	5	2	26
	454	6	3	38
	455	9	4	50
	456	11	5	64
	457	5	6	77

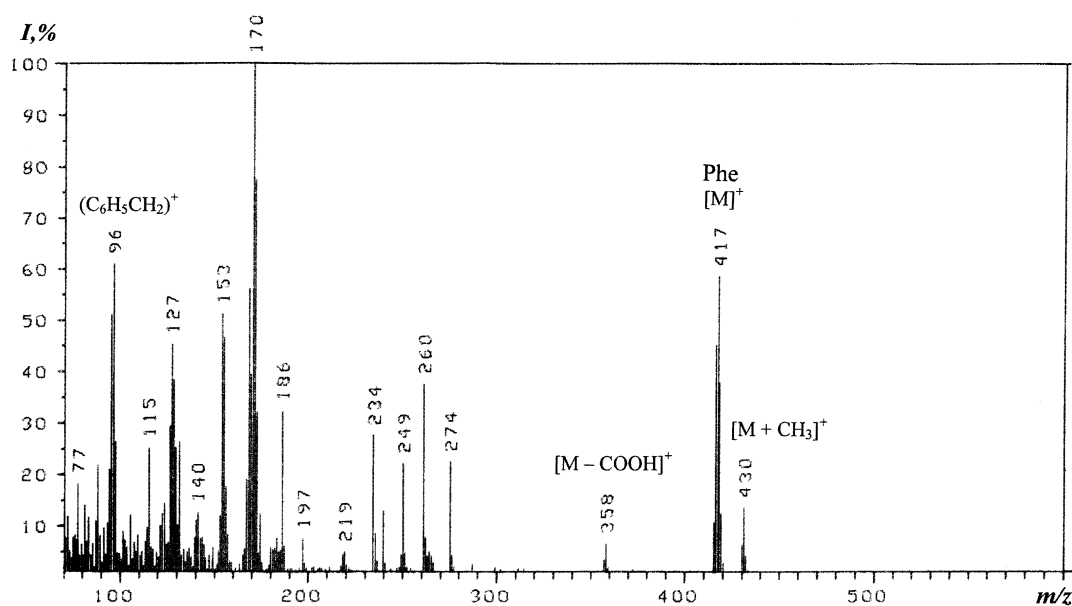
Notes:

\* A dash means no incorporation of deuterium.

\*\* In calculating the level of deuterium enrichment protons(deuterons) at carboxyl and NH<sub>2</sub>-amino groups of amino acids were not considered due to the easily isotopic (H-<sup>2</sup>H) exchange.



a)



b)

Figure 4. EI mass spectra of methyl N-Dns-[2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]Phe under various experimental conditions: a) – the unlabeled methyl ester of N-Dns-Phe; b) – methyl ester of N-Dns-[2, 3,4,5,6-<sup>2</sup>H<sub>5</sub>]Phe, isolated from the BR hydrolyzate by RP HPLC. Separation conditions: 250 × 10 mm Column C18 (“Kova”, Slovakiya), eluents: (A) – CH<sub>3</sub>CN–CF<sub>3</sub>COOH (100: 0.1–0.5% (v/v)) and (B) – CH<sub>3</sub>CN (100% (v/v)).

Data for chromatographic separation of methyl N-Dns-derivatives of [<sup>2</sup>H]amino acids from different natural sources do not differ from those for methyl esters of unlabeled N-Dns-amino acid derivatives, as the isotopic enrichment of molecules virtually has no effect on chromatographic characteristics. The method was adapted to the conditions of the chromatographic separation of a mixture of methyl esters of N-Dns-[<sup>2</sup>H]derivatives of amino acids hydrolyzate of BR, consisting in the optimization of the ratio of eluent, form and rates of gradient elution from the column. In addition, while using a gradient the maximum number of peaks in the chromatogram significantly increases – peak capacity, which is very important for the separation of complex multicomponent mixtures, which are the protein hydrolysates. In this case, each component of the mixture is separated under the most optimal composition of the eluent, thereby achieving their full separation quality in much less time than with the isocratic regime. The method was adapted to the conditions of chromatographic separation of a mixture of methyl esters of N-Dns-[<sup>2</sup>H]amino derivatives of BR hydrolyzate comprising in optimization of eluent ratios, the gradient type, and the rate of gradient elution from the column. The best separation was achieved by gradient elution with a solvent mixture – CH<sub>3</sub>CN–CF<sub>3</sub>COOH (100: 0,1–0,5% (v/v)) (A) and (B) – CH<sub>3</sub>CN (100% (v/v)) by progressively increasing the concentration of component B in the mixture from 10 to 100%. Samples were dissolved in 10 ml MeOH and applied in an amount of 50-100 μl on a column (250×10 mm) with octadecylsilane silica gel Separon SGX C 18,7 μm. Herewith it was possible to isolate the tryptophan and inseparable pair phenylalanine/tyrosine. The levels of chromatographic purity of methyl esters of N-Dns-[2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]phenylalanine, N-Dns-[3,5-<sup>2</sup>H<sub>2</sub>]tyrosine and N-Dns-[2, 4,5,6,7-<sup>2</sup>H<sub>5</sub>]tryptophan

isolated from protein hydrolysate were 89, 91 and 90%, respectively with the output 97–85%. The result confirmed Figure 4b which shows the EI mass spectrum of methyl N-Dns-[2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]phenylalanine isolated by RP-HPLC (scanning at  $m/z$  70–600, base peak at  $m/z$  170, 100%) (EI mass spectrum is shown relative to the unlabeled methyl ester of N-Dns-phenylalanine (Figure 4a), scanning at  $m/z$  150–700, base peak at  $m/z$  250, 100%). Proof for the inclusion of 5 deuterium atoms into phenylalanine molecule is the presence of “heavy” molecular ion peak of methyl ester of N-Dns-[<sup>2</sup>H]phenylalanine ( $[M]^+$  at  $m/z$  417, 59%, instead of  $[M]^+$  at  $m/z$  412, 44% for non-labeled derivative of phenylalanine), and the additional deuterium-enriched peaks of benzyl C<sub>6</sub>H<sub>5</sub>CH<sub>2</sub> fragments of [<sup>2</sup>H]phenylalanine molecule at  $m/z$  96, 61% (instead of  $m/z$  91, 55% in the control (not shown)) (Fig. 4b). Peaks of secondary fragments of varying intensity with values at  $m/z$  249, 234 and 170 are the secondary decomposition products of the dansyl residue to the 5-(dimethylamino)naphthalene; low intensity peak  $[M - \text{COOCH}_3]^+$  at  $m/z$  358, 7% (instead at  $m/z$  353, 10% in the control) is a product of cleavage of a carboxymethyl (COOCH<sub>3</sub>) group of the methyl ester of N-Dns-[<sup>2</sup>H]phenylalanine, and a peak  $[M + \text{CH}_3]^+$  at  $m/z$  430, 15% (instead of  $m/z$  426, 8% in the control) – is the product of further methylation of  $\alpha$ -amino group in the [<sup>2</sup>H]phenylalanine molecule (Fig. 4b). According to the EI mass spectrometry data, the difference between the molecular weight of protonated and deuterated species calculated by the peaks  $[M]^+$  of methyl ester of N-Dns-phenylalanine makes up 5 units that corresponds with the primary data on the deuterium enrichment level of [2,3,4,5,6-<sup>2</sup>H<sub>5</sub>]phenylalanine added to the growth medium. Mass spectrometric data on deuterium enrichment levels of [3,5-<sup>2</sup>H<sub>2</sub>]tyrosine and [2,4,5,6,7-<sup>2</sup>H<sub>5</sub>]tryptophan are also in good correlation and confirmed by <sup>1</sup>H NMR.

#### 4. Conclusion

The experimental data indicate the high efficiency of the of incorporation of H-labeled aromatic amino acids into the BR molecule with output of BR 8–10 mg per 1 g of biomass. The main advantage of this method is that the isolated BR keeps its native configuration in combination with 13-*trans*-retinal, and the ability to photochemical reactions *in vitro*, as demonstrated by electrophoresis in 12.5% (w/v) PAAG with 0.1% (w/v) SDS-Na. The method is also applicable for the preparation of other similar to BR transmembrane proteins of halophilic bacteria – sensory rhodopsin and halorhodopsin. The unique properties of natural bacteriorhodopsins provide a wide range of bio- and nano-technological applications in which may find their application <sup>2</sup>H-labeled analogues. In the future we plan obtaining fully deuterated analogs of BR for the reconstruction of functionally active systems of transmembrane proteins in heavy water with purified <sup>2</sup>H-labeled fatty acids and other biologically active compounds. These studies will provide an answer to the question of how function BR in the native membranes under condition of the complete replacement of protons by deuterium.

#### References

Atanasov, A., Karadzov, S., Ivanova, E., Mosin, O. V. & Ignatov, I. (2014) Study of the Effects of Electrochemical Aqueous Sodium Chloride Solution (Anolite) on the Virus of Classical Swine Fever Virus. Mathematical Models of Anolyte and Catolyte as Types of Water, *Journal of Medicine, Physiology and Biophysics*, 4: 1-26.



- Gui-Ying Ch., Chun-Ping Zh., Zong-Xia G., Jian-Guo T., Guang-Yin Zh. & Qi-Wang S (2012) All-optical logic-gates based on bacteriorhodopsin film. *Advanced Studies in Biology*, 4(5), 207–216.
- Hampp N. & Oesterhelt D. (2004) Bacteriorhodopsin and its potential in technical applications, in *Nanobiotechnology*, Eds. Ch. Niemeyer & C. Mirkin. Weinheim: Wiley-VCH-Verlag, pp. 130–136.
- Haupt U., Tittor J., Bamberg E. & Oesterhelt D. (1997) General concept for ion translocation by halobacterial retinal proteins: the isomerization/switch/transfer model. *Biochemistry*, 36(2–7), 78–85.
- Ignatov, I., Antonov, A. & Galabova, T. (1998) Medical Biophysics – Biophysical Fields of Man, *Gea Libris*, Sofia.
- Ignatov, I., Antonov, A. & Galabova, T., (2002) Scientific Research Studies with Christos Drossinakis (October 2001 – October 2002), *Int. Conference “Man and Nature”*, SRCMB, Sofia.
- Ignatov, I. (2005) Energy Biomedicine, *Gea-Libris*, Sofia: 1–88.
- Ignatov, I. (2007) There are not Reliable Results with Research with Infrared Spectroscopy of Homeopathic Solutions after Avogadro’s Number, *Ministry of Health*, 196-199. [in Russian]
- Marinov, M., Ignatov, I. (2008) Color Kirlian Spectral Analysis. Color Observation with Visual Analyzer, *Euromedica*, Hanover, 57-59
- Ignatov, I. (2010) Which water is optimal for the origin (generation) of life? *Euromedica*, Hanover: 34-35.
- Ignatov, I. (2011) Entropy and time in living matter, *Euromedica*: 74.
- Ignatov, I., Tsvetkova, V. (2011) Water for the Origin of Life and “Informationability” of Water, Kirlian (Electric Images) of Different Types of Water, *Euromedica*, Hanover: 62-65.
- Ignatov, I. (2012) Origin of Life and Living Matter in Hot Mineral Water, Conference on the Physics, Chemistry and Biology of Water, *Vermont Photonics*, USA.
- Ignatov, I., & Mosin, O.V. (2012) Isotopic Composition of Water and its Temperature in Modeling of Primordial Hydrosphere Experiments, VIII Intern. Conference Perspectives of the Development of Science and Technique, *Biochemistry and Biophysics*, **15**: 41–49.
- Ignatov, I. & Mosin, O. V. & Naneva, K. (2012) Water in the Human Body is Information Bearer about Longevity, *Euromedica*, Hanover: 110-111.
- Ignatov, I. & Mosin, O. V. (2013) Method for Color Coronal (Kirlian) Spectral Analysis, Biomedical *Radio electronics*, *Biomedical Technologies and Radio electronics*, **3**: 38-47. [in Russian]
- Ignatov I. & Mosin O.V. (2013) Possible Processes for Origin of Life and Living Matter with Modeling of Physiological Processes of Bacterium *Bacillus Subtilis* in Heavy Water as Model System, *Journal of Natural Sciences Research*, **3** (9): 65-76.
- Ignatov, I. & Mosin, O. V. (2013) Modeling of Possible Processes for Origin of Life and Living Matter in Hot Mineral and Seawater with Deuterium, *Journal of Environment and Earth Science*, **3**(14): 103-118.
- Ignatov, I., Mosin, O. V. (2013) Structural Mathematical Models Describing Water Clusters, *Journal of Mathematical Theory and Modeling*, **3** (11): 72-87.
- Ignatov, I. & Mosin, O.V. (2014) Photoreceptors in Visual Perception and Additive Color Mixing. Bacteriorhodopsin in Nano- and Biotechnologies, *Advances in Physics Theories and Applications*, Vol. 27, pp. 20 -37.
- Ignatov, I. & Mosin, O. V. (2014) The Structure and Composition of Carbonaceous Fullerene Containing Mineral Shungite and Microporous Crystalline Aluminosilicate Mineral Zeolite. Mathematical Model of

- Interaction of Shungite and Zeolite with Water Molecules *Advances in Physics Theories and Applications*, **28**: 10-21.
- Ignatov, I., Mosin, O.V., Velikov, B., Bauer, E. & Tyminski, G. (2014) Longevity Factors and Mountain Water as a Factor. Research in Mountain and Field Areas in Bulgaria, *Civil and Environmental Research*, **6** (4): 51-60.
- Ignatov, I., Mosin, O. V., Niggli, H.&Drossinakis, Ch. (2014) Evaluating Possible Methods and Approaches for Registering of Electromagnetic Waves Emitted from the Human Body, *Advances in Physics Theories and Applications*, **30**: 15-33.
- Ignatov, I., Mosin, O.V.&Drossinakis, Ch. (2014) Infrared Thermal Field Emitted from Human Body. Thermovision, *Journal of Medicine, Physiology, Biophysics*, **1**:1-12.
- Ignatov, I., Mosin, O.V.&Velikov, B. (2014) Longevity Factors and Mountain Water of Bulgaria in Factorial Research of Longevity, *Journal of Medicine, Physiology, Biophysics*,**1**:13-33.
- Ignatov, I.&Mosin,O.V. (2014) Visual Perception. Electromagnetic Conception for the Eyesight. Rhodopsin and Bacteriodopsin, *Journal of Medicine, Physiology and Biophysics*, **2**:1-19.
- Ignatov, I.&Mosin,O.V. (2014) The Structure and Composition of Shungite and Zeolite. Mathematical Model of Distribution of Hydrogen Bonds of Water Molecules in Solution of Shungite and Zeolite, *Journal of Medicine, Physiology and Biophysics*, **2**: 20-36.
- Ignatov, I., Mosin,O.V., Velikov, B., Bauer, E.&Tyminski, G. (2014) Research of Longevity Factors and Mountain Water as a Factor in Teteven Municipality, Bulgaria, *Journal of Medicine, Physiology and Biophysics*, **2**: 37-52.
- Ignatov, I.&Mosin,O.V. (2014) Modeling of Possible Processes for Origin of Life and Living Matter in Hot Mineral Water. Research of Physiological Processes of Bacterium Bacillus Subtilis in Hot Heavy Water, *Journal of Medicine, Physiology and Biophysics*, **2**: 53-70.
- Ignatov, I.&Mosin,O.V. (2014) Mathematical Models of Distribution of Water Molecules Regarding Energies of Hydrogen Bonds, *Journal of Medicine, Physiology and Biophysics*, **2**: 71-94.
- Ignatov, I.&Mosin,O.V. (2014) Modeling of Possible Conditions For Origin of First Organic Forms in Hot Mineral Water, *Journal of Medicine, Physiology and Biophysics*, **3**: 1-14.
- Ignatov, I.&Mosin,O.V. (2014) Mathematical Model of Interaction of Carbonaceous Fullerene Containing Mineral Shungite and Aluminosilicate Mineral Zeolite with Water, *Journal of Medicine, Physiology and Biophysics*, **3**: 15-29.
- Ignatov, I. &Mosin,O.V. (2014) Studying of Phototransformans of Light Signal by Photoreceptor Pigments – Rhodopsin, Iodopsin and Bacteriorhopsin and Additive Mixing of Colors, *Journal of Medicine, Physiology and Biophysics*, **3**: 30-47.
- Ignatov, I. &Mosin,O.V. (2014) Mathematical Models Describing Water Clusters as Interaction among Water Molecules. Distributions of Energies of Hydrogen Bonds, *Journal of Medicine, Physiology and Biophysics*, **3**: 48-70.
- Ignatov, I., Mosin, O. V.&Bauer, E. (2014) Carbonaceous Fullerene Mineral Shungite and Aluminosilicate Mineral Zeolite. Mathematical Model and Practical Application of Water Solution of Water Shungite and Zeolite, *Journal of Medicine, Physiology and Biophysics*, **4**: 27-44.

- Ignatov, I. & Mosin, O. V. (2014) Nature of Hydrogen Bonds in Liquids and Crystals. Ice Crystal Modifications and Their Physical Characteristics, *Journal of Medicine, Physiology and Biophysics*, **4**: 58-80.
- Ignatov, I. & Mosin, O. V. (2014) Research of Isotopic Effects of Deuterium in Cells of Microorganisms in the Presence of D<sub>2</sub>O in IR Spectra in Hot Mineral Water for Origin of Life, *Journal of Medicine, Physiology and Biophysics*, **4**: 45-57.
- Ignatov, I. & Mosin, O. V. (2014) Visual Perception and Electromagnetic Conception for the Eyesight. Rhodopsin and Bacteriorhodopsin in Nano- and Biotechnologies, *Journal of Health, Medicine and Nursing*, **4**: 1-20.
- Ignatov, I., Mosin, O.V., Velikov, B., Bauer, E. & Tyminski, G. (2014) Research of Longevity Factors and Mountain Water as a Factor in Teteven, Yablanitsa and Ugarchin Municipalities, Lovech Region, Bulgaria, *Journal of Health, Medicine and Nursing*, **4**: 21-36.
- Ignatov, I. Mosin, O.V., Velikov, B. Bauer, E. & Tyminski, G. (2014) Mountain Water as Main Longevity Factor in Research of Phenomenon of Longevity in Mountain Areas in Bulgaria, *European Journal of Molecular Biotechnology*, **4** (2): 52-71.
- Ignatov, I., Mosin, O. V., Niggli, H., Drossinakis, Ch. & Stoyanov, Ch. (2014) Registration of Electromagnetic Waves Emitted the Human Body, *Journal of Medicine, Physiology and Biophysics*, **5**: 1-22.
- Ignatov, I. & Mosin, O. V. (2014) Modeling of Possible Processes for Origin of Life and Living Matter in Sea and Hot Mineral Water. Process of Formation of Stromatolites, *Journal of Medicine, Physiology and Biophysics*, **5**, pp. 23-46.
- Ignatov, I., Mosin, O. V. (2014) Coronal Gas Discharge Effect in Modeling of Non-Equilibrium Conditions with Gas Electric Discharge Simulating Primary Atmosphere and Hydrosphere for Origin of Life and Living Matter, *Journal of Medicine, Physiology and Biophysics*, **5**: 47-70.
- Ignatov, I., Mosin, O. V. & Bauer, E. (2014) Mathematical Model and Practical Application of Water Solution of Shungite and Zeolite. Composition of Aluminosilicate Mineral Zeolite and Carbonaceous Fullerene Containing Mineral Shungite, *Journal of Health, Medicine and Nursing*, **5**: 12-29.
- Ignatov, I. & Mosin, O. V. (2014) Hydrogen Bonds among Molecules in Liquid and Solid State of Water. Modifications of Ice Crystals, *Journal of Health, Medicine and Nursing*, **5**: 56-79.
- Ignatov, I. & Mosin, O. V. (2014) Microorganisms in the Presence of D<sub>2</sub>O and IR Spectra in Hot Mineral Water with More Deuterium Atoms for Origin of Life and Living Matter, *Journal of Health, Medicine and Nursing*, **5**: 80-93.
- Ignatov, I. & Mosin, O.V. (2014) Color Coronal (Kirlian) Spectral Analysis in Modeling of Nonequilibrium Conditions with the Gas Electric Discharges, Simulating Primary Atmosphere, *Biomedical Radioelectronics*, **2**: 42-51. [in Russian]
- Ignatov, I., Mosin, O.V. & Stoyanov, Ch. (2014) Biophysical Fields. Color Coronal Spectral Analysis. Registration with Water Spectral Analysis. Biophoton Emission, *Journal of Medicine, Physiology and Biophysics*, **6**: 1-22.
- Ignatov, I. & Mosin, O.V. (2014) Hot Mineral Water with More Deuterium for Origin of Live and Living Matter. Process of Formation of Stromatolites, *Journal of Health, Medicine and Nursing*, **6**: 1-24.

- Ignatov, I. & Mosin, O.V. (2014) Origin of Life and Living Matter in Primary Atmosphere and Hydrosphere. Modeling of Non-equilibrium Electric Gas Discharge Conditions, *Journal of Health, Medicine and Nursing* **6**: 25-49
- Ignatov, I. & Mosin, O.V. (2014) Methods for Measurements of Water Spectrum. Differential Non-equilibrium Energy Spectrum Method (DNES), *Journal of Health, Medicine and Nursing* **6**: 50-72.
- LeMaster D.M. (1990) Uniform and selective deuteration in two-dimensional NMR studies of proteins. *Ann. Rev. Biophys. Chem.*, **19**, 243–266.
- Karadzov, S., Atanasov, A., Ivanova, E., Mosin, O. V. & Ignatov, I. (2014) Mathematical Models of Electrochemical Aqueous Sodium Chloride Solutions (Anolyte and Catolyte) as Types of Water. Study of the Effects of Anolyte on the Virus of Classical Swine Fever Virus, *Journal of Health, Medicine and Nursing*, **5**: 30-55.
- Liu T.Y. & Chang Y.H. (1971) Hydrolysis of proteins with p-toluenesulfonic acid. Determination of tryptophan. *J. Biol. Chem.*, **246**(9), 2842–2848.
- Mosin O.V., Karnaukhova E.N., Pshenichnikova A.B. & Reshetova O.S. (1994) Electron impact mass-spectrometry in bioanalysis of stable isotope labeled bacteriorhodopsin, in 6th Intern. Conf. on Retinal proteins, Leiden: Elsevier, pp. 115–116.
- Mosin O.V, Skladnev D.A., Egorova T.A. & Shvets V.I. (1996) Mass spectrometric evaluation of the level switch in  $^2\text{H}$  and  $^{13}\text{C}$  amino acid molecules of bacterial objects. *Bioorganic Chemistry*, **22**(10–11), 856–869.
- Mosin O.V., Skladnev D.A. & Shvets V.I. (1999) Inclusion of deuterated aromatic amino acids in the molecule of bacteriorhodopsin *Halobacterium halobium*. *Applied Biochemistry and Microbiology*, **35**(1), 34–42.
- Mosin O.V., Shvets V.I., Skladnev D.A. & Ignatov I. (2012) Synthesis of [ $^2\text{H}$ ]bacteriorhodopsin labeled by deuterium on residues of aromatic amino acids. *Khimicheskaya Tekhnologiya (Chemical Engineering)*, Moscow: Publishing House “Nauka & Technology”, **9**, 553–564.
- Mosin O.V., Shvets V.I., Skladnev D.A. & Ignatov I. (2013) Biosynthesis of trans-membrane photo-transforming protein [ $^2\text{H}$ ]bacteriorhodopsin, labeled with deuterium on residues of aromatic amino acids [ $2,3,4,5,6\text{-}^2\text{H}_5$ ]Phe, [ $3,5\text{-}^2\text{H}_2$ ]Tyr and [ $2,4,5,6,7\text{-}^2\text{H}_5$ ]Trp. *Problems of Biological, Medical and Pharmaceutical Chemistry*, **8**, 29–39.
- Mosin O.V. & Ignatov I. (2012) Isotope effects of deuterium in bacterial and microalgae cells at growth on heavy water ( $\text{D}_2\text{O}$ ). *Voda: Ximia i Ecologija*, **3**, 83–94 [in Russian].
- Mosin O.V. & Ignatov I. (2013) Microbiological synthesis of  $^2\text{H}$ -labeled phenylalanine, alanine, valine, and leucine/isoleucine with different degrees of deuterium enrichment by the Gram-positive facultative methylotrophic bacterium *Brevibacterium methylicum*. *International Journal of Biomedicine*, **3**(2), 132–138.
- Mosin, O.V. & Ignatov, I. (2014) Improved of Method for Isolation of Photochrome Transmembrane Protein Bacteriorhodopsin from Purple Membranes of Halobacterium Halobacterium Halobium, *Journal of Medicine, Physiology and Biophysics*, **3**: 71-86.

- Mosin, O.V. & Ignatov, I. (2014) Photochrome Transmembrane Protein Bacteriorhodopsin from Purple Membranes of Halobacterium Halobium in Nano- and Biotechnologies, *Journal of Medicine, Physiology and Biophysics*, **4**: 81-99.
- Mosin, O.V. & Ignatov, I. (2014) Facultative Methylotrophic Bacterium Brevibacterium Methylicum in the Process of Preparation of Highly Deuterated Phenylalanine, Alanine, Valine and Leucine/Isoleucine, *Journal of Health, Medicine and Nursing*, **4**: 37-56.
- Mosin, O.V. & Ignatov, I. (2014) Isolation of Photochrome Transmembrane Protein Bacteriorhodopsin from Purple Membranes of Halobacterium Halobacterium Halobium, New Method for Isolation, *Journal of Health, Medicine and Nursing*, **4**: 57-73.
- Mosin, O.V. & Ignatov, I. (2014) Research from a Bacterium Bacillus Subtilis B-3157 by FAB Method of Biosynthetic Pathways of 2H-Labeled Purine Ribonucleoside Inosine, *Journal of Health, Medicine and Nursing*, **4**: 74-93.
- Mosin, O. V. & Ignatov, I. (2014) Phenomenon of Biological Adaptation to Deuterium Oxide, *Journal of Medicine, Physiology and Biophysics*, **5**: 71-107.
- Mosin, O. V. & Ignatov, I. (2014) The Strain of Facultative Methylotrophic Bacterium Brevibacterium Methylicum 5662 with RuMP Cycle of Carbon Assimilation for Biosynthesis of Deuterium Labeled Phenylalanine, *Journal of Medicine, Physiology and Biophysics*, **5**: 108-128.
- Mosin, O. V. & Ignatov, I. (2014) Photochrome Transmembrane Protein Bacteriorhodopsin from Purple Membrane of Halobacterium Halobium. Applications in Bio- and Nanotechnologies, *Journal of Health, Medicine and Nursing*, **5**: 94-112.
- Mosin, O.V. & Ignatov, I. (2014) Biosynthesis of Deuterium-labeled Transmembrane Protein Bacteriorhodopsin using a Halobacterium Halobium, *Journal of Medicine, Physiology and Biophysics*, **6**: 23-41.
- Mosin, O.V. & Ignatov, I. (2014) Photochrome Protein Bacteriorhodopsin Protein from Purple Membranes of Halobacterium Halobium. Application Applications in Bio- and Nanotechnologies, *Journal of Medicine, Physiology and Biophysics*, **6**: 42-60.
- Mosin, O.V. & Ignatov, I. (2014) Metabolism and Physiology of Methylotrophic Microorganisms, *Journal of Medicine, Physiology and Biophysics*, **6**: 61-84.
- Mosin, O.V. & Ignatov, I. (2014) On the Nature of Biological Adaptation on Deuterium Oxide, *Journal of Medicine, Physiology and Biophysics*, **6**: 85-121.
- Mosin, O.V. & Ignatov, I. (2014) Phenomenon of Biological Adaptation to Heavy Water, *Journal of Health, Medicine and Nursing*, **6**: 73-110.
- Mosin, O.V. & Ignatov, I. (2014) Biosynthesis of Deuterium Labeled Phenylalanine. Facultative Methylotrophic Bacterium Brevibacterium Methylicum #5662 with RuMP Cycle of Carbon Assimilation, *Journal of Health, Medicine and Nursing*, **6**: 111-132.
- Mosin, O.V. & Ignatov, I. (2014) Basic Concept of Magnetic Water Treatment, *European Journal of Molecular Biotechnology*, **4** (2): 72-85.
- Neugebauer D.Ch., Zingsheim H.P. & Oesterhelt D. (1978) Recrystallization of the purple membrane *in vivo* and *in vitro*". *Journal Molecular Biology*, 123, 247-257.
- Nonella M., Windemuth A. & Schulten K. (1991) Structure of bacteriorhodopsin and *in situ* isomerization of retinal: a molecular dynamics study. *Journal Photochem. Photobiol.*, 54(6), 937-948.

Oesterhelt D. & Stoeckenius W. (1971) Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature*, 233(89), 149–160.

Oesterhelt D. (1988) The structure and mechanism of the family of retinal proteins from halophilic Archaea. *Struct. Biol.*, 8, 489–500.

Penke B., Ferenczi R. & Kovács K. (1974) A new acid hydrolysis method for determining tryptophan in peptides and proteins. *Analytical Biochemistry*, 60(1), 45–50.

Pshenichnikova A.B., Karnauhova E.N., Zvonkova E.N. & Shvets V.I. (1995) Methods for preparation of deuterated amino acids. *Bioorganic Chemistry*, 21(3), 163–178.

Rudiger M., Tittor J., Gerwert K. & Oesterhelt D. (1997) Reconstitution of bacteriorhodopsin from the apoprotein and retinal studied by Fourier-transformed infrared spectroscopy. *Biochemistry*, 36, 4867–4874.

Vought B.W. & Birge R.R. (1999) Molecular electronics and hybrid computers, in *Wiley Encyclopedia of Electrical and Electronics Engineering*, Eds. B.W. Vought & R.R. Birge. New York: Wiley-Interscience, pp. 224–240.

Zvonkova E.N., Zotchik N.V., Philippovich E.I., Mitrofanov T.K., Myagkova G.I. & Serebrennikova G.A. (1970) *Chemistry of Biologically Active Natural Compounds*. Moscow: Chemistry, pp. 68–80 [in Russian].