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Biological Influence of Deuterium on Procariotic and Eukaryotic Cells

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Abstract. Biologic influence of deuterium (D) on cells of various taxonomic groups of prokaryotic and eukaryotic microorganisms realizing methylotrophic, chemoheterotrophic, photo-organotrophic, and photosynthetic ways of assimilation of carbon substrates are investigated at growth on media with heavy water (D_2O). The method of step by step adaptation technique of cells to D_2O was developed, consisting in plating of cells on 2 % agarose nutrient media containing increasing gradient of concentration of D_2O (from 0 up to 98 % D_2O) and the subsequent selection of stable to D_2O cells. In the result of that technique were obtained adapted to maximum concentration of D_2O cells, biological material of which instead of hydrogen contained deuterium with levels of enrichment 92–97,5 at.% D.

Keywords: deuterium; heavy water; adaptation; isotopic effects; bacteria; blue-green algae.

Introduction.

The most interesting biological phenomenon is the ability of some microorganisms to grow on heavy water (D_2O) media in which all hydrogen atoms are replaced with deuterium [1, 2]. D_2O has high environmental potential due to the absence of radioactivity and poccebility of detecting the deuterium label in the molecule by high-resolution methods as NMR, IR, and mass spectrometry that facilitates its use as a tracer in biochemical and biomedical research [3]. The average ratio of H/D in nature makes up approximately 1:5700 [4]. In mixtures of D_2O with H_2O the isotopic exchange occurs with high speed with the formation of semi-heavy water (HDO): $D_2O + H_2O = HDO$. For this reason deuterium presents in smaller content in aqueous solutions in form of HDO, while in the higher content – in form of D_2O.

The chemical structure of D_2O molecule is analogous to that one for H_2O, with small differences in the length of the covalent H–O-bonds and the angles between them. The molecular mass of D_2O exceeds on 10 % that one for H_2O. The difference in nuclear masses stipulates the isotopic effects, which may be sufficiently essential for H/D pair [5]. As a result, physical-chemical properties of D_2O differ from H_2O: D_2O boils at 101,44 ^{0}C, freezes at 3,82 ^{0}C, has maximal density at 11,2 ^{0}C (1,106 g/cm^3) [6]. The chemical reactions in D_2O are somehow slower compared to H_2O.

D_2O is less ionized, the dissociation constant of D_2O is smaller, and the solubility of the organic and inorganic substances in D_2O is smaller compared to these ones in H_2O [7]. Due to isotopic effects the hydrogen bonds with the participation of deuterium are slightly stronger than those ones formed of hydrogen.

For a long time it was considered that heavy water is incompatible with life. Experiments with the growing of cells of different organisms in D_2O show toxic influence of deuterium. The high concentrations of D_2O lead to the slowing down the cellular metabolism, mitotic inhibition of the prophase and in some cases – somatic mutations [8]. This is observed even while using natural water with an increased content of D_2O or HDO [9]. Bacteria can endure up to 90 % (v/v) D_2O, plant cells can develop normally up to 75 % (v/v) D_2O, while animal cells – up to not more than 30 % (v/v) D_2O [10]. The decrease of the deuterium content in water to 25 % (v/v) of the physiological level stimulates the cellular metabolism.

With the development of new microbiological approaches, there appears an opportunity to use adapted to deuterium cells for preparation of deuterated natural compounds [11, 12]. The traditional method for production of deuterium labelled compounds consists in the growth on media containing maximal concentrations of D_2O and deuterated substrates as [D]methanol, [D]glucose etc. [13]. During growth of cells on D_2O are synthesized molecules of biologically important natural compounds (DNA, proteins, amino acids, nucleosides, carbohydrates, fatty acids), which hydrogen atoms at the carbon backbones are completely substituted with deuterium. They are being isolated from deuterated biomass obtained on growth media with high D_2O content and deuterated substrates with using a combination of physico-chemical methods of separation – hydrolysis, precipitation and extraction with organic solvents and chromatographic purification by column chromatography on different adsorbents. These deuterated molecules evidently undergo structural adaptation modifications necessary for the normal functioning in D_2O.

The adaptation to D_2O is interested not only from scientific point, but allows to obtain the unique biological material for the studying of molecular structure by ^{1}H-NMR [14]. Trend towards the use of deuterium as an isotopic label are stipulated by the absence of radioactivity and possebility of determination the deuterium localization in the molecule by high resolution NMR spectroscopy [15], IR spectroscopy [16] and mass spectrometry [17]. The recent advances in the technical and computing capabilities of analytical methods have allowed to considerable increase the efficiency of de novo biological studies, as well as to carry out structural-functional studies with deuterated molecules on a molecular level.

This study is a continuation of our research for the practical utilization of different cells of bacteria and microalgae for the synthesis of deuterium labeled compounds in deuterated growth media with D_2O. The purpose of our research was studying the physiological influence of deuterium on the cells of different taxonomic groups of microorganisms and microalgae realizing methylotrophic, chemoheterotrophic, photo-organotrophic and photosynthetic pathways of carbon assimilation.

Material and methods.

The objects of the study were various microorganisms, realizing methylotrophic, chemoheterotrophic, photo-organotrophic, and photosynthetic ways of assimilation of carbon substrates. The initial strains were obtained from the State Research Institute of Genetics and Selection of Industrial Microorganisms (Moscow, Russia):

1. *Brevibacterium methylicum* B-5652, a leucine auxotroph Gram-positive strain of facultative methylotrophic bacterium, L-phenylalanine producer, assimilating methanol via the NAD^+ dependent methanol dehydrogenase variant of ribulose-5-monophosphate cycle (RuMP) of carbon fixation.

2. *Bacillus subtilis* B-3157, a polyauxotrophic for histidine, tyrosine, adenine, and uracil spore-forming aerobic Gram-positive chemoheterotrophic bacterium, inosine producer, realizing hexose-6-mono-phosphate (GMP) cycle of carbohydrates assimilation.

3. *Halobacterium halobium* ET-1001, photo-organotrophic carotenoid-containing strain of extreme halobacteria, synthesizing the phototransforming transmembrane protein bacteriorhodopsin.

4. *Chlorella vulgaris* B-8765, photosynthesizing single-cell blue-green algae.

For preparation of growth media was used D_2O (99,9 at.%) and DCl (95,5 at.%) received from the "Isotope" Russian Research Centre (St. Petersburg, Russian Federation). Inorganic salts and glucose were preliminary crystallized in D_2O and dried in vacuum before using. D_2O distilled over $KMnO_4$ with the subsequent control of isotope enrichment by ^{1}H-NMR-spectroscopy on a Brucker WM-250 device ("Brucker", Germany) (working frequency 70 MHz, internal standard Me_4Si).

For cell cultivation and adaptation were used various growth media with an increasing gradient of D_2O concentration from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) D_2O. Cultivation of methylotrophic bacteria was carried out on minimal salt M9 medium (g/l): KH_2PO_4 – 3; Na_2HPO_4 – 6; NaCl – 0,5; NH_4Cl – 1. Cultivation of chemoheterotrophic bacteria was carried out on HW medium (g/l): glucose – 12; yeast extract or hydrolyzed deuterated biomass of *B. methylicum* – 2,5; NH_4NO_3 – 3; $MgSO_4 \cdot 7H_2O$ – 2; $CaCO_3$ – 2. Cultivation of photo-organotrophic bacteria was carried out on SM medium (g/l): yeast extract or hydrolyzed deuterated biomass of *B. methylicum* – 2,5; NaCl – 250; $MgSO_4 \cdot 7H_2O$ – 20; KCl – 2; NH_4Cl – 0,5; KNO_3 – 0,1; KH_2PO_4 – 0,05; K_2HPO_4 – 0,05; Na^+-citrate – 0,5; $MnSO_4 \cdot 2H_2O$ – $3 \cdot 10^{-4}$; $CaCl_2 \cdot 6H_2O$ – 0,065; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $FeSO_4 \cdot 7H_2O$ – $5 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$; glycerol – 1,0. Blue-green algae *C. vulgaris* grew on mineral growth medium (g/l): KNO_3 – 5,0; $MgSO_4 \cdot 7H_2O$ – 2,5; KH_2PO_4 – 1,25; $FeSO_4$ – 0,003; $MnSO_4 \cdot 2H_2O$ – $3 \cdot 10^{-4}$; $CaCl_2$ $6H_2O$ – 0,065; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$, $CoCl_2 \cdot 6H_2O$ – $5 \cdot 10^{-6}$.

For adaptation were used solid 2 % (w/v) agarose media with gradually increasing concentration of D_2O, combined with the subsequent selection of individual cellular colonies resistant to deuterium. As a source of deuterated growth substrates for the growth of chemoheterotrophic bacteria and photo-organotrophic bacteria was used the deuterated biomass of facultative methylotrophic bacterium *B. methylicum*, obtained via a multi-stage adaptation on solid 2 % (w/v) agarose M9 media with an increasing gradient of D_2O (from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) D_2O). Raw deuterated biomass (output, 100 gram of wet weight per 1 liter of liquid culture) was suspended in 100 ml 0,5 M ^{2}HCl (in D_2O) and autoclaved for 30–40 min at 0,8 atm. The suspension was neutralized with 0,2 M KOH (in D_2O) to pH = 7,0 and used as a source of growth substrates for growing of chemoheterotrophic bacterium *B. sublilis* and photo-organotrophic bacterium *H. halobium*.

Cellular growth was carried out in 500 ml Erlenmeyer flasks containing 100 ml of the growth medium at 32–34 ^{0}C and vigorously aerated on an orbital shaker Biorad ("Biorad Labs", Poland). Photo-organotrophic bacteria and blue-green algae were grown at illumination by fluorescent monochromatic lamps LDS-40-2 (40 W) ("Alfa-Electro", Russia). Growing of microalgae *C. vulgaris* was carried out at 32 ^{0}C in a photoreactor with CO_2 bubbling. Bacterial growth was monitored on ability to form individual colonies on a surface of 2 % (w/v) agarose media, and on optical density of cell suspension measured on Beckman DU-6 (Beckman Coulter, USA) spectrophotometer at λ = 620 nm. After 6–7 days the cells were harvested and separated by centrifugation (10000 g, 20 min) on T-24 centrifuge ("Heracules", Germany). The biomass was washed with D_2O and extracted with a mixure of organic solvents: chloroform-methanol-acetone = 2: 1: 1, % (v/v) for isolating lipids and pigments. The resulting precipitate (10–12 mg) was dried in vacuum and used as a protein fraction, while the liquid extract – as a lipid fraction. The exogenious deuterated amino acids and ribonucleosides were isolated from the liquid cultures of appropriate strain-producers. Inosine was isolated from LC of *B. subtilis* by adsorption/desorption on activated carbon as adsorbent with following extraction with 0,3 M NH_4-formate buffer (pH = 8,9), subsequent crystallization in 80 % (v/v) ethanol, and ion exchange chromatography (IEC) on a column with cation exchange resin AG50WX 4 equilibrated with 0,3 M NH_4-formate buffer and 0,045 M NH_4Cl (output, 3,1 g/l (80 %); $[\alpha]_D^{20}$ = 1,61 (ethanol)). Bacteriorhodopsin was isolated from the purple membranes of photo-organotrophic halobacterium *H. halobium* by the method of D. Osterhelt, modificated by the authors [18].

The analysis of amino acids from protein hydrolyzates was carried out on a Biotronic LC-5001 (230 × 3,2) column ("Eppendorf–Nethleler–Hinz", Germany) with a UR-30 ("Beckman–Spinco", USA) sulfonated styrene (7,25 % cross linked) resin as a stationary phase; the granule diameter was 25 μm; 0,2 N sodium–citrate buffer (pH = 2,5) was used as an eluent; the working pressure – 50–60 atm; the eluent input rate – 18,5 ml/h; the ninhydrin input rate – 9,25 ml/h; detection at λ = 570 and λ = 440 nm (for proline).

Carbohydrates were analyzed on a Knauer Smartline chromatograph ("Knauer", Germany) equipped with a Gilson pump ("Gilson Inc.", Germany) and a Waters K-401 refractometer ("Water Associates", Germany) using Ultrasorb CN as a stationary phase: the column size – 250 × 10 mm; the granule diameter – 10 μm; the mobile phase – acetonitrile–water (75 : 25, % (w/w); the input rate – 0,6 ml/min.

Fatty acids were analyzed on a Beckman Gold System (USA) chromatograph, equipped with Model 126 Detector (USA). Stationary phase: Ultrasphere ODS, particel size 5 μm, 4,6 × 250 mm; mobile phase: linear gradient of 5 mM KH_2PO_4-acetonitrile, elution rate 0,5 ml/min, detection at λ = 210 nm.

For evaluation of deuterium enrichment levels EI and FAB mass spectrometry was used. EI mass spectra were recorded on MB-80A device ("Hitachi", Japan) with double focusing (the energy of ionizing electrons, 70 eV; the accelerating voltage, 8 kV; the cathode temperature, 180–200 ^{0}C) after amino acid modification into methyl esters of N-5-dimethylamino(naphthalene)-1-sulfonyl (dansyl) amino acid derivatives according to an earlier elaborated protocol [19]. FAB-mass spectra were recorded on pulse mass spectrometer VG-70 SEQ ("Fisons VG Analytical", USA), supplied with caesium source Cs^+ on a glyceric matrix with accelerating pressure 5 кВ and an ionic current 8 мА.

IR-spectra were registered on Brucker Vertex ("Brucker", Germany) IR spectrometer (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}).

Results and discussion.

When biological objects being exposed to water with different deuterium content, their reaction varies depending on the isotopic composition of water and magnitude of isotope effects determined by the difference of constants of chemical reactions rates k_H/k_D in H_2O and D_2O. The maximum kinetic isotopic effect observed at ordinary temperatures in chemical reactions leading to rupture of bonds involving hydrogen and deuterium lies in the range k_H/k_D = 5–**7** for C–H versus C–D, N–D versus N–D, and O–H versus O–D-bonds. Isotopic effects have an impact not only on the physical and chemical properties of macromolecules, but also on the biological behaviour of biological objects in D_2O. Experiments with D_2O (Table 1) have shown, that green-blue algae is capable to grow on 70 % (v/v) D_2O, methylotrophic bacteria – 75 % (v/v) D_2O, chemoheterotrophic bacteria – 82 % (v/v) D_2O, and photo-organotrophic halobacteria – 95 % (v/v) D_2O.

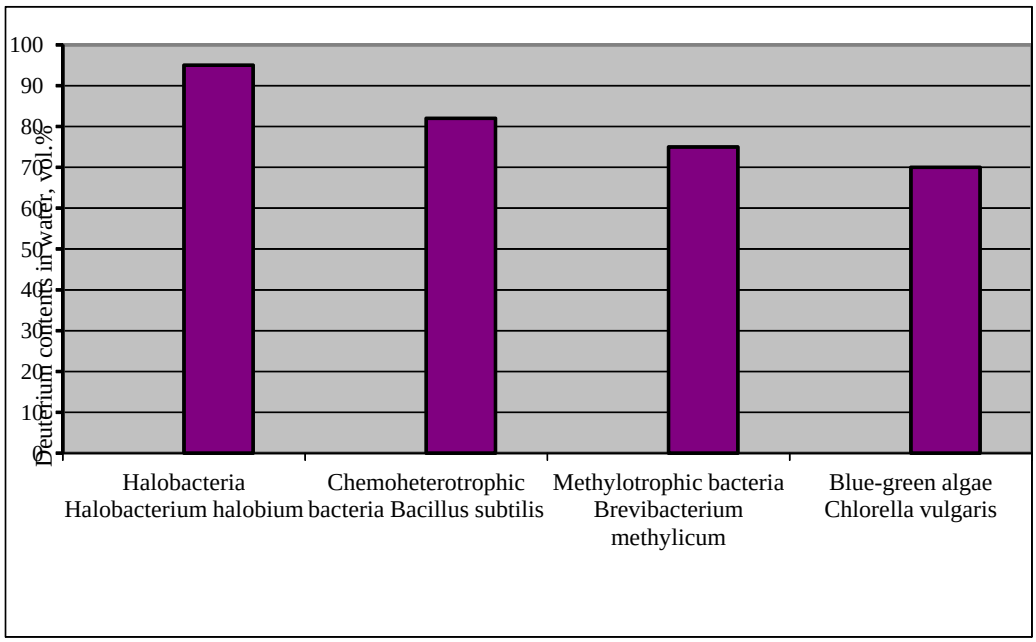

Figure 1. Cell survival of studied microorganisms in water with different deuterium content

In the course of the experiment were obtained adapted to the maximum concentration of D_2O cells belonging to different taxonomic groups of microorganisms, realizing methylotrophic, chemoheterotrophic, photo-organotrophic and photosynthetic pathways of assimilation of carbon substrata, as facultative methylotrophic bacterium *B. methylicum*, chemoheterotrophic bacterium *B. subtilis*, halobacterium *H. halobium* and blue-green algae *C. vulgaris*.

Selection of methanol-assimilating facultative methylotrophic bacterium *B. methylicum* was connected with the development of new microbiological strategies for preparation of deuterated biomass via bioconversion of [D]methanol and D_2O and its further use as a source of deuterated growth substrates for the growing of other strains-producers in D_2O.

Choosing of photo photo-organotrophic halobacteria *H. halobium* was stipulated by the prospects of further isolation of retinal containing transmembrane protein bacteriorhodopsin (BR) – chromoprotein of 248 amino acid residues, containing as a chromophore an equimolar mixture of 13-*cis*-and 13-*trans* C20 carotenoid associated with a protein part of the molecule via a Lys-216 residue [20]. BR performs in the cells of halobacteria the role of ATP-dependent translocase, which creates 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.

Using chemoheterotrophic bacterium *B. subtilis* was determined by preparative isolation produced by this bacterium deuterated ribonucleoside – inosine (total deuteration level 65,5 at.% D) for biomedical use [21], and the use of photosynthetic blue-green *C. vulgaris* was stipulated by the study of biosynthesis of deuterated chlorophyll and carotenoid pigments (deuteration level 95–97 at.% D) on growth media with high D_2O-content.

We used stepwise increasing gradient concentration of D_2O in growth media, because it was assumed that the gradual accustoming of micorganisms to deuterium would have a beneficial effect upon the growth and physiological parameters. The strategy of adaptation to D_2O is shown in Table. 1 on an example of methylotrophic bacterium *B. methylicum*, which deuterated biomass was used in further experiments as a source of deuterated growth substrates for growing of chemoheterotrophic and photo-organotrophic bacteria.

Table 1

The isotopic composition of growth media and growth characteristics of methylotrophic bacterium *B. methylicum* in the process of adaptation to D_2O*

Experiment number	Media components, % (v/v)		Lag-period, (h)	Yeald of biomass, gram from 1 liter of liquid culture	Cell generation time (h)
	H_2O	D_2O			
1	98,0	0	20,0±1,40	200,02±1.40	20,0±1,40
2	73,5	24,5	34,0±0,89	171,8±1.81	2,6±0,23
3	49,0	49,0	44,0±1,38	121,3±1.83	3,2±0,36
4	24,5	73,5	49,0±0,91	94,4±1.74	3,8±0,25
5	0	98,0	60,0±2,01	60,2±1.44	4,9±0,72
6	0	98,0	40,0±0,88	174,0±1.83	2,8±0,30

Notes: * The data in Expts. 1–5 is submitted for *B. methylicum* at growing on growth media, containing 2 % (v/v) deutero-methanol and specified amounts (% , v/v) of D_2O.
The data in Expt. 6 is submitted for adapted to D_2O bacterium.
As the control used experiment 1 where used protonated water and methanol.

The adaptation strategy to D_2O consisted in plating of initial cells of microorganisms on Petri dishes with solid 2 % (w/v) agarose growth media with stepwise increasing D_2O-content therein (0; 24,5; 49,0; 73,5 and 98 % (v/v) D_2O), and the subsequent selection of resistant cells to D_2O. Cells grown on media with a low gradient of D_2O concentration were consequently transferred onto media with higher gradient, up to 98 % (v/v) D_2O. At the final stage of this procedure on the maximally deuterated growth medium with 98 % (v/v) D_2O were isolated individual cell colonies representing the progeny of a single cell resistant to the action of D_2O. Then the colonies were transferred onto the liquid growth medium of the same D_2O-content, prepared on the basis of 98 % (v/v) D_2O and grown for 5 days at 34 ^{0}C. The survival rate in the maximal deuterated growth

medium was not more than 40 %. The progress of adaptation was observed by the changes of lag-time period, time of cell generation and yrald of microbial biomass, as well as by the ability of cells to form single colonies on the surface of solid 2 % (w/v) agarose media with ^2H_2O and cell counting.

All microorganisms adapted to D_2O retained the ability to grow on growth media with high content of D_2O. The general feature of bacterial growth in D_2O was the proportional increase in duration of the lag-period and time of cellular generation and simultaneous reduction of yealds of microbial biomass. These parameter values were correlated with the content of D_2O in growth media with the lowest fixing values of these parameters on maximum deuterated media. The added gradually increasing concentrations of D_2O into growth media caused the proportional increasing lag-period and output of microbial biomass in all isotopic experiments (Table 1). In contrast to the adapted microorganisms, the growth of non-adapted microorganisms on the maximal deuterated media with D_2O was inhibited. The yealds of biomass on deuterated growth media were varied 85–90 % for different taxonomic groups of microorganisms. Adapted microorganisms possessed slightly reduced levels of microbial biomass accumulation and increased cell generation times on maximal deuterated media.

The result obtained in experiments on the adaptation of methylotrophic bacterium *B. methylicum* to D_2O allowed to use hydrolysates of biomass of this bacterium obtained in the process of multi-stage adaptation to D_2O, as a source of deuterated growth substrates for the growing of chemoheterotrophic bacterium *B. subtillis* and photo-organotrophic halobacterium *H. halobium*. The assimilation rate of methylotrophic biomass by protozoa and eukaryotic cells amounts to 85–98 %, while the productivity calculated on the level of methanol bioconversion into cell components makes up 50–60 % [22]. While using deutereted biomass of methylotrophic bacteria *B. methylicum* as a sourse of deuterated growth substrates it was taken into account the ability of methylotrophic bacteria to synthesize large amounts of protein (output, 50 % (w/w) of dry weight), 15–17 % (w/w) of polysaccharides, 10–12 % (w/w) of lipids (mainly, phospholipids), and 18 % (w/w) of ash [23]. The most important fact is that ability is preserved on growth media containing D_2O and [D]methanol. To provide high outputs of these compounds and minimize the isotopic exchange (H–D) in amino acid residues of protein molecules, the biomass was hydrolyzed by autoclaving in 0,5 M DCl (in D_2O) and used for the growing of chemoheterotrophic bacterium *B. subtillis* and photo-organotrophic halobacterium *H. halobium*.

Taking into account the pathways of assimilation of carbon substrates, the adaptation of chemoheterotrophic bacterium *B. subtilis* and photo-organotrophic halobacterium *H. halobium* was carried out *via* plating of initial cells to separate colonies on solid 2 % (w/v) agarose media based on 99,9 at.% D_2O and deuterated hydrolyzate biomass of *B. methylicum*, with the following subsequent selection of the colonies resistant to D_2O. On contrary to D_2O deuterated substrates in composition of deuterated biomass hydrolyzate had no significant negative effect on the growth parameters of the studied microorganisms. Output of deuterated inosine at growth of *B. subtilis* on D_2O-medium was 3,9 g/l, while the level of glucose assimilation from the liquid culture was 40 g/l. The fractionation of inosine from liquid culture was performed by adsorption/desorption on the surface of activated carbon, extraction by 0,3 M NH_4-formate buffer (pH = 8,9) with subsequent crystallization in 80 % (v/v) ethanol and column ion exchange chromatography on cation exchange resin AG50WX 4 equilibrated with 0,3 M NH_4-formate buffer with 0,045 M NH_4Cl. Deuteration level of inosine molecule meadsured by FAB mass spectrometry was five deuterium atoms (62,5 atm.% D) with the inclusion of three deuterium atoms in the ribose and two deuterium atoms in the molecule fragments hypoxanthine.

For adaptation of blue-green algae *C. vulgaris* was used liquid mineral medium containing 25, 50, 75 and 98 % (v/v) D_2O. The levels of deuterium enrichment of carotenoids were In the case of *C. vulgaris* and *H. halobium* used fluorescent illumination, as both microorganisms grown in the presence of light. Individual colonies of cells of these microorganisms resistant to D_2O, allocated by selection were grown on liquid growth media of the same composition with 99,9 at.% D_2O for producing the deuterated biomass.

While growing of photo-organotrophic halobacterium *H. halobium* on D_2O-medium cells synthesized the purple carotenoid pigment, identified as a native BR on the the spectral ratio of protein and chromophore fragments in the molecule (D_{280}/D_{568} = 1,5 : 1,0). The growth of this bacterium on D_2O-medium was slightly inhibited as compared with the control on protonated

growth medium that simplifies the optimization of conditions for the production of microbial biomass, which consists in the growing of this halobacterium on deuterated growth medium with 2 % (w/v) of deuterated biomass hydrolyzate of *B. methylicum*, isolation of purple membrane fraction, the separation of low- and high-molecular impurities, cellular RNA, pigments (preferably carotenoids) and lipids, protein solubilization in 0,5 % (w/v) SDS-Na, fractionation of solubilized protein by methanol and purification on Sephadex G-200. The total level of deuterium enrichment of the BR molecule, calculated on deuterium enrichment levels of amino acids of the protein hydrolyzate was 95 at.% D.

Our studies indicated that the ability of adaptation to D_2O for different taxonomic groups of microorganisms is different, and stipulated by taxonomic affiliation, metabolic characteristics, pathways of assimilation of substrates, as well as by evolutionary niche occupied by the object. Thus, the lower the level of evolutionary organization of the organism, the easier it adapted to the presence of deuterium in growth media. Thus, most primitive in evolutionary terms (cell membrane structure, cell organization, resistance to environmental factors) of the studied objects are photo-organotrophic halobacteria related to archaebacteria, standing apart from both prokaryotic and eukaryotic microorganisms, exhibiting increased resistance to ^2H_2O and practically needed no adaptation to D_2O, contrary to blue-green algae, which, being eukaryotes, are the more difficult adapted to D_2O and exhibit inhibition of growth on 70–75 % (v/v) D_2O.

The composition of growth media evidently plays an important role in process of adaptation to D_2O, because the reason of inhibition of cell growth and cell death can be changes of the parity ratio of synthesized metabolites in D_2O-media: amino acids, proteins and carbohydrates. It is noted that adaptation to D_2O occures easier on complex growth media than on the minimal growth media with full substrates at a gradual increasing of deuterium content in the growth media, as the sensitivity to D_2O of different vital systems is different. As a rule, even highly deuterated growth media contain remaining protons from 0,2–10 at.%. These remaining protons facilitate the restructuring to the changed conditions during the adaptation to D_2O, presumably integrating into those sites, which are the most sensitive to the replacement of hydrogen by deuterium. Also deuterium induces physiological, morphological and cytological alterations on the cell. There were marked the significant differences in the morphology of the protonated and deuterated cells of blue-green algae *C. vulgaris*. Cells grown on D_2O-media were 2–3 times larger in size and had thicker cell walls, than the control cells grown on a conventional protonated growth media with ordinary water, the distribution of DNA in them was non-uniform. In some cases on on the surface of cell membranes may be observed areas consisting of tightly packed pleats of a cytoplasmic membrane resembling mezosoms – intracytoplasmic bacterial membrane of vesicular structure and tubular form formed by the invasion of cytoplasmic membrane into the cytoplasm (Fig. 2). It is assumed that mezosoms involved in the formation of cell walls, replication and segregation of DNA, nucleotides and other processes. There is also evidence that the majority number of mezosoms being absent in normal cells is formed by a chemical action of some external factors – low and high temperatures, fluctuation of pH and and other factors. Furthermore, deuterated cells of *C. vulgaris* were also characterized by a drastic change in cell form and direction of their division. The observed cell division cytodieresis did not end by the usual divergence of the daughter cells, but led to the formation of abnormal cells, as described by other authors [24]. The observed morphological changes associated with the inhibition of growth of deuterated cells were stipulated by the cell restructuring during the process of adaptation to D_2O. The fact that the deuterated cells are larger in size (apparent size was of 2–4 times larger than the size of the protonated cells), apparently is a general biological phenomenn proved by growing a number of other adapted to D_2O prokaryotic and eukaryotic cells.

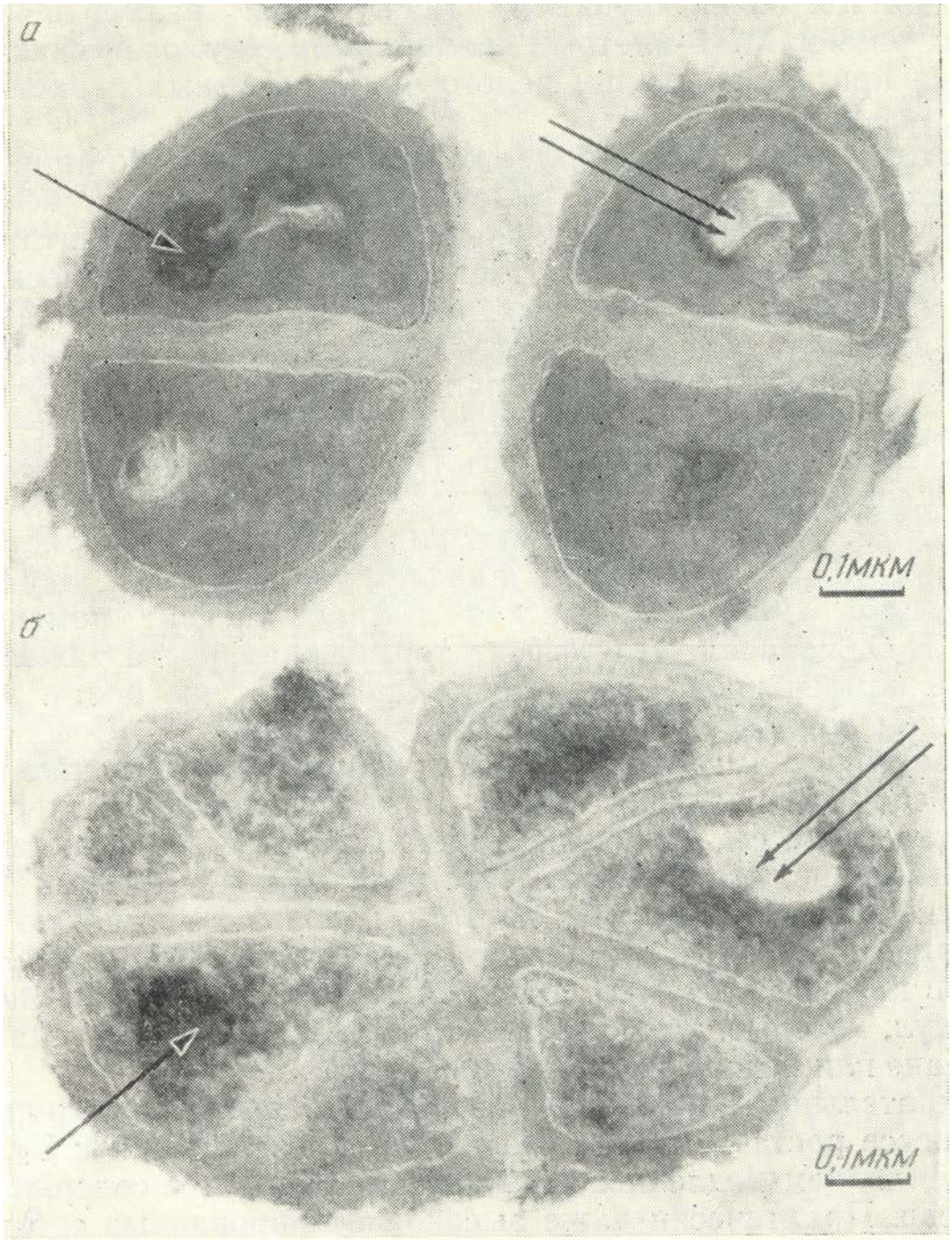

Figure 2. Electron micrographs of *Micrococcus lysodeikticus* cells: *a*) – protonated cells obtained on H_2O-medium; *b*) – deuterated cells obtained on D_2O-medium [24]. The arrows show the tightly-packed portions of the membranes

Our data generally confirm a stable notion that adaptation to D_2O is a phenotypic phenomenon as the adapted cells eventually return back to the normal growth after some lag-period after their replacement back onto H_2O-medium. At the same time the effect of reversibility of growth on H_2O/D_2O does not exclude an opportunity that a certain genotype determines the displaying of the same phenotypic attribute in D_2O-media with maximum deuterium content. At placing a cell onto D_2O-media lacking protons, not only H_2O is removed from a cell due to isotopic (H–D) exchange, but also there are occurred a rapid isotopic (H–D) exchange in hydroxyl (-OH), *sulfohydryl* (-SH) and amino ($-NH_2$) groups in all molecules of organic substances, including proteins, nucleic acids, carbohydrates and lipids. It is known, that in these conditions only covalent C–H bond is not exposed to isotopic (H–D) exchange and, thereof only molecules with bonds such as C–D can be synthesized de novo [25]. Depending on the position of the

deuterium atom in the molecule, there are distinguished primary and secondary isotopic effects mediated by intermolecular interactions. In this aspect, the most important for the structure of macromolecules are dynamic short-lived hydrogen (deuterium) bonds formed between the electron deficient H(D) atoms and adjacent electronegative O, C, N, S- heteroatoms in the molecules, acting as acceptors of H-bond. The hydrogen bond, based on weak electrostatic forces, donor-acceptor interactions with charge-transfer and intermolecular van der Waals forces, is of the vital importance in the chemistry of intermolecular interactions and maintaining the spatial structure of macromolecules in aqueous solutions. Another important property is defined by the three-dimensional structure of D_2O molecule having the tendency to pull together hydrophobic groups of macromolecules to minimize their disruptive effect on the hydrogen (deuterium)-bonded network in D_2O. This leads to stabilization of the structure of protein and nucleic acid macromolecules in the presence of D_2O. That is why, the structure of macromolecules of proteins and nucleic acids in the presence of D_2O is somehow stabilized [26].

Evidently the cell implements a special adaptive mechanisms promoting the functional reorganization of vital systems in D_2O. Thus, for the normal synthesis and function in D_2O of such vital compounds as nucleic acids and proteins contributes to the maintenance of their structure by forming hydrogen (deuterium) bonds in the molecules. The bonds formed by deuterium atoms are differed in strength and energy from similar bonds formed by hydrogen. Somewhat greater strength of D–O bond compared to H–O bond causes the differences in the kinetics of reactions in H_2O and D_2O. Thus, according to the theory of a chemical bond the breaking up of covalent H–C bonds can occur faster than C–D bonds, the mobility of D_3O^+ ion is lower on 28,5 % than H_3O^+ ion, and OD^- ion is lower on 39,8 % than OH^- ion, the constant of ionization of D_2O is less than that of H_2O [27]. These chemical-physical factors lead to a slow down in the rates of enzymatic reactions in D_2O [28]. However, there are also such reactions which rates in D_2O are higher than in H_2O. In general these reactions are catalyzed by D_3O^+ or H_3O^+ ions or OD^- and OH^- ions. The substitution of H with D affects the stability and geometry of hydrogen bonds in an apparently rather complex way and may, through the changes in the hydrogen bond zero-point vibration energies, alter the conformational dynamics of hydrogen (deuterium)-bonded structures of DNA and proteins in D_2O. It may cause disturbances in the DNA-synthesis, leading to permanent changes on DNA structure and consequently on cell genotype. Isotopic effects of deuterium, which would occur in macromolecules of even a small difference between hydrogen and deuterium, would certainly have the effect upon the structure. The sensitivity of enzyme function to the structure and the sensitivity of nucleic acid function (genetic and mitotic) would lead to a noticeable effect on the metabolic pathways and reproductive behaviour of an organism in the presence of D_2O. And next, the changes in dissociation constants of DNA and protein ionizable groups when transferring the macromolecule from H_2O to D_2O may perturb the charge state of the DNA and protein molecules. All this can cause variations in nucleic acid synthesis, which can lead to structural changes and functional differences in the cell and its organelles. Thus, the structural and dynamic properties of the cell membrane, which depends on qualitative and quantitative composition of membrane fatty acids, can also be modified in the presence of D_2O. The cellular membrane in the bacteria is one of the most important organelles for metabolic regulation, combining apparatus of biosynthesis of polysaccharides, transformation of energy, supplying cells with nutrients and involvement in the biosynthesis of proteins, nucleic acids and fatty acids. Obviously, the cell membrane plays an important role in the adaptation to D_2O. But it has been not clearly known what occurs with the membranes – how they react to the replacement of H to D and how it concerns the survival of cells in D_2O-media devoid of protons.

Comparative analysis of the fatty acid composition of deuterated cells of chemoheterotrophic bacteria *B. subtilis*, obtained on the maximum deuterated medium with 99,9 at.% D_2O, carried out by HPLC method, revealed significant quantitative differences in the fatty acid composition compared to the control obtained in ordinary water (Fig. 3 *a, b*). Characteristically, in a deuterated sample fatty acids having retention times at 33,38; 33,74; 33,26 and 36,03 min are not detected in HPLC-chromatogram (Fig. 3*b*). This result is apparently due to the fact that the cell membrane is one of the first cell organelles, sensitive to the effects of D_2O, and thus compensates the changes in rheological properties of a membrane (viscosity, fluidity, structuredness) not only by quantitative but also by qualitative composition of membrane fatty acids. Similar situation was observed with

the separation of other natural compounds (proteins, amino acids, carbohydrates) extracted from deutero-biomass obtained from maximally deuterated D_2O-medium.

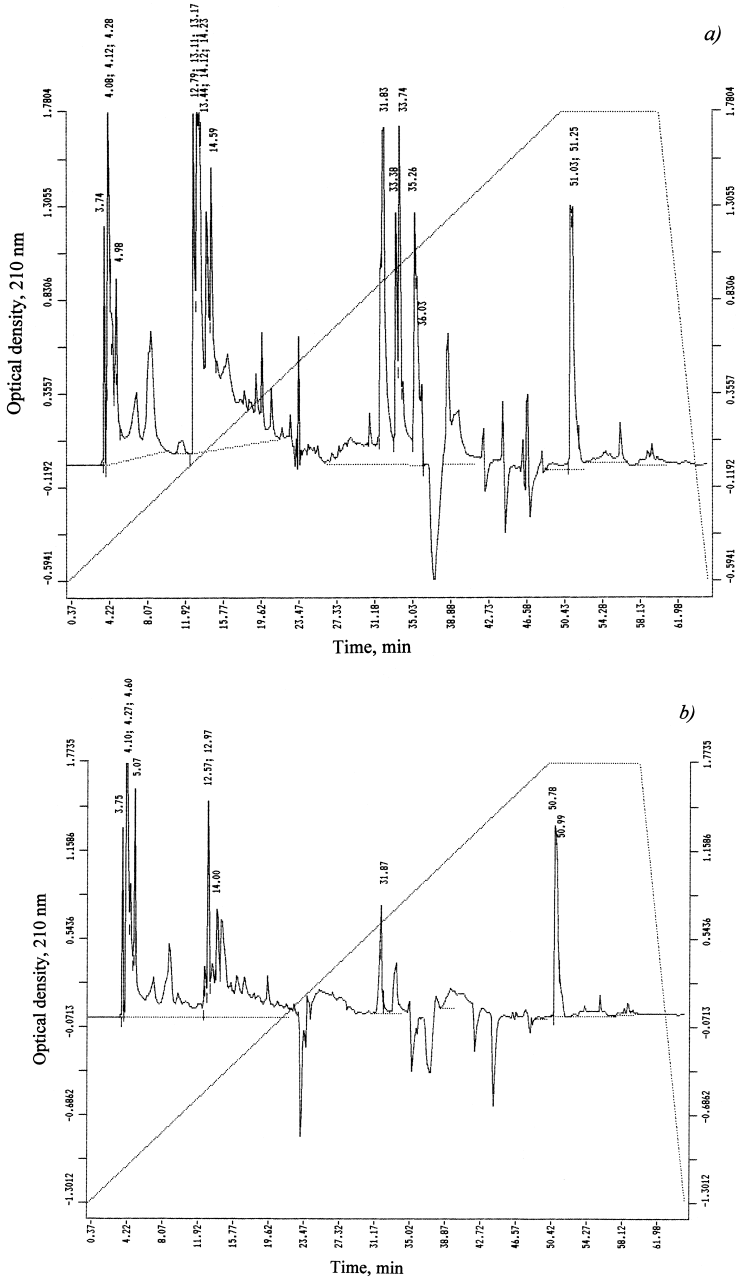

Figure 3. HPLC-chromatograms of fatty acids obtained from protonated (*a*) and deuterated (*b*) cells *B. subtilis* on the maximally deuterated D_2O-medium. The peaks on chromatograms with retention time 3,75 min (instead of 3,74 minutes in the control), 4,10; 4,27; 4,60 (instead of 4,08; 4,12; 4,28 in the control), 5,07 (instead of 4,98 in control) 12,57; 12,97 (instead of 12,79; 13,11; 13,17 in control) 14,00 (instead of 14,59 in the control), 31,87 (instead of 31,83 in the control); 33,38; 33,74; 33,26; 36,03; 50,78; 50,99 (instead of 51,03; 51,25 for control) correspond to individual intracellular fatty acids

Amino acid analysis of protein hydrolysates and intracellular carbohydrates isolated from deuterated cells of *B. subtilis*, also revealed the differences in quantitative composition of amino acids synthesized in D_2O-medium. Protein hydrolyzates contains fifteen identified amino acids (except proline, which was detected at λ = 440 nm) (Table 2). An indicator that determines a high efficiency of deuterium inclusion into amino acid molecules of protein hydrolyzates are high levels of deuterium enrichment of amino acid molecules, which are varied from 50 at.% for leucine/isoleucine to 97,5 at.% for alanine

Table 2

Amino acid composition of the protein hydrolysates of *B. subtilis*, obtained on the maximum deuterated medium and levels of deuterium enrichment of molecules*

Amino acid	Yield, % (w/w) dry weight per 1 gram of biomass		Number of deuterium atoms incorporated into the carbon backbone of a molecule**	Level of deuterium enrichment of molecules, % of the total number of hydrogen atoms***
	Protonated sample (control)	The sample obtained in 99,9 % D_2O		
Glycine	8,03	9,69	2	90,0
Alanine	12,95	13,98	4	97,5
Valine	3,54	3,74	4	50,0
Leucine	8,62	7,33	5	50,0
Isoleucine	4,14	3,64	5	50,0
Phenylalanine	3,88	3,94	8	95,0
Tyrosine	1,56	1,83	7	92,8
Serine	4,18	4,90	3	86,6
Threonine	4,81	5,51	–	–
Methionine	4,94	2,25	–	–
Asparagine	7,88	9,59	2	66,6
Glutamic acid	11,68	10,38	4	70,0
Lysine	4,34	3,98	5	58,9
Arginine	4,63	5,28	–	–
Histidine	3,43	3,73	–	–

Notes:

* The data obtained by mass spectrometry for the methyl esters of N-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl) amino acid derivatives.

** While calculating the level of deuterium enrichment protons (deuterons) at the carboxyl (COOH-) and NH_2-groups of amino acid molecules are not taken into account because of their easy dissociation in H_2O/D_2O

*** A dash means absence of data.

Qualitative and quantitative composition of the intracellular carbohydrates of *B. subtilis* obtained on maximally deuterated D_2O-medium shown in Table. 3 (the numbering is given to the sequence of their elution from the column) contained monosaccharides (glucose, fructose, rhamnose, arabinose), disaccharides (maltose, sucrose), and four other unidentified carbohydrates with retention time 3,08 min (15,63 %); 4,26 min (7,46 %); 7,23 min (11,72 %) and 9,14 min (7,95 %) (not shown). Yield of glucose in deuterated sample makes up 21,4 % by dry weight, i.e. higher than for fructose (6,82 %), rhamnose (3,47 %), arabinose (3,69 %), and maltose (11,62 %). Their outputs are not significantly different from control in H_2O except for sucrose in deuterated sample that was not detected (Table 3). The deuterium enrichment levels of carbohydrates were varied from 90,7 at.% for arabinose to 80,6 at.% for glucose.

Table 3

Qualitative and quantitative composition of intracellular carbohydrates of *B. subtilis* obtained on the maximally deuterated medium and levels of deuterium enrichment of molecules*

Carbohydrate	Content in the biomass, % of the dry weight of 1 g biomass		Level of deuterium enrichment, % of the total number of hydrogen atoms***
	Protonated sample (control)	The sample obtained in 99,9 % D_2O**	
Glucose	20,01	21,40	80,6
Fructose	6,12	6,82	85,5
Rhamnose	2,91	3,47	90,3
Arabinose	3,26	3,69	90,7
Maltose	15,30	11,62	–
Sucrose	8,62	ND	–

Notes:
* The data were obtained by IR-spectroscopy.
** ND – not detected
** A dash means the absence of data.

Conclusions.

Our experiments demonstrated that the effects observed at the cellular growth on D_2O possess a complex multifactor character connected to changes of physiological parameters – magnitude of the lag-period, time of cellular generation, outputs of biomass, a ratio of amino acids, protein, carbohydrates and fatty acids synthesized in D_2O, and with an evolutionary level of organization of investigated object as well. The cell evidently implements the special adaptive mechanisms promoting functional reorganization of work of the vital systems in the presence of D_2O. Thus, the most sensitive to replacement of H on D are the apparatus of biosynthesis of macromolecules and a respiratory chain, i.e., those cellular systems using high mobility of protons and high speed of breaking up of hydrogen bonds. Last fact allows the consideration of adaptation to D_2O as adaptation to the nonspecific factor affecting simultaneously the functional condition of several numbers of cellular systems: metabolism, ways of assimilation of carbon substrates, biosynthetic processes, and transport function, structure and functions of macromolecules. It seems to be reasonable to choose as biomodels in these studies microorganisms, as they are very well adapted to the environmental conditions and able to withstand high concentrations of D_2O in the growth media.

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Биологическое влияние дейтерия на клетки прокариот и эукариот

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Аннотация. Изучено биологическое воздействие дейтерия (D) на клетки различных таксономических групп прокариотических и эукариотических микроорганизмов, реализующих метилотрофные, хемогетеротрофные, фото-органогетеротрофные и фотосинтетические способы ассимиляции углеродных субстратов при росте на средах с тяжёлой водой (D_2O). Разработан метод ступенчатой адаптации клеток к D_2O, заключающийся в их рассеве на чашках Петри с твердыми (2 %-ный агар) питательными средами при ступенчатом увеличении градиента концентрации D_2O (от 0 до 98 % D_2O) и последующей селекции устойчивых к D_2O клеток. В результате этой техники на максимально дейтерированной среде с 98 % D_2O получены адаптированные к D_2O клетки, биологический материал которых вместо атомов водорода содержит атомы дейтерия с уровнем дейтерированности молекул 92–97,5 ат.% D.

Ключевые слова: дейтерий; тяжелая вода; изотопные эффекты; адаптация; бактерии; сине-зеленые водоросли.