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# Biological Influence of Deuterium on Prokaryotic 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 was investigated at cellular growth on media with maximal content of heavy water ($D_2O$). For this aim the method of step by step adaptation technique of cells to $D_2O$ was developed, consisting in plating of cells on 2 % (w/v) agarose nutrient media containing increasing gradient of concentration of $D_2O$ (from 0 up to 98 % (v/v) $D_2O$) and the subsequent selection of stable to $D_2O$ cells. Cells grown on media with a low gradient of $D_2O$ concentration were consequently transferred onto media with higher gradient of $D_2O$, up to 98 % (v/v) $D_2O$. In the result of that technique were isolated individual cell colonies representing the progeny of a single cell resistant to the action of $D_2O$, biological material of which instead of hydrogen contained deuterium with high levels of deuterium enrichment ~92.0–97.5 atom.% D. Our studies demonstrated that the effects observed at the cellular growth on $D_2O$ possess a complex multifactor character connected to changes of cytological, morphological and physiological parameters – cellular size, non-uniform distribution of DNA, 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. The maximum kinetic isotopic effect observed at 22 $^0$C in biochemical reactions leading to rupture of bonds involving H and D atoms 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.

**Keywords:** bacteria; blue-green algae, deuterium; heavy water; isotopic effects, IR-spectroscopy.

## 1. 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 (Ignatov & Mosin, 2013a; Ignatov & Mosin, 2013b). $D_2O$ has high environmental potential in biomedical studies 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 (Kushner *et al*., 1999).

The average ratio of H/D in nature makes up approximately 1:5700 (Lis *et al*., 2008). In natural waters, the deuterium content is distributed irregularly: from 0.02–0.03 mol.% for river and sea water, to 0.015 mol.% for water of Antarctic ice – the most purified from deuterium natural water containing deuterium in 1.5 times less than that of seawater. According to the international SMOW standard isotopic shifts for D and $^{18}$O in sea water: D/H = $(155.76 \pm 0.05)\cdot 10^{-6}$ (155.76 ppm) and $^{18}O/^{16}O = (2005.20 \pm 0.45)\cdot 10^{-6}$ (2005 ppm). For SLAP standard isotopic shifts for D and $^{18}$O in seawater: D/H = $89\cdot 10^{-6}$ (89 ppm) and for a pair of

$^{18}O/^{16}O = 1894 \cdot 10^{-6}$ (1894 ppm). In surface waters, the ratio D/H = ~$(1.32–1.51) \cdot 10^{-4}$, while in the coastal seawater – ~$(1.55–1.56) \cdot 10^{-4}$. The natural waters of CIS countries are characterized by negative deviations from SMOW standard to $(1.0–1.5) \cdot 10^{-5}$, in some places up to $(6.0–6.7) \cdot 10^{-5}$, but there are observed positive deviations at $2.0 \cdot 10^{-5}$.

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 (Lobishev & Kalinichenko, 1978). 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$^3$) (Vertes, 2004). 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 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 $H_2O$ (Mosin, 1996). 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 (Den'ko, 1970). This is observed even while using natural water with an increased content of $D_2O$ or HDO (Stom *et al*., 2006). 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) $D_2O$ (Mosin & Ignatov, 2012a). Further increase in the concentration of $D_2O$ for these groups of organisms leads to cellular death (Katz, 1960; Thomson, 1960), although cell's cultures suspended in pure $D_2O$ exert a strong radioprotective effect in $D_2O$-solutions towards γ–radiation (Michel *et al*., 1988; Laeng *et al*., 1991). On the contrary, deuterium depleted water with decreased deuterium content has benefitial effects on organism and stimulates the cellular metabolism (Somlyai, 2001; Sinyak *et al*., 2003).

With the development of new microbiological approaches, there appears an opportunity to use adapted to deuterium cells for preparation of deuterated natural compounds (Mosin *et al*., 2013a; Mosin *et al*., 2013b; Mosin *et al*., 2013c). 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. (Mosin & Ignatov, 2012b; Mosin *et al*., 2014). 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 (Crespi, 1989). 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 (LeMaster, 1990), IR spectroscopy (MacCarthy, 1985) and mass spectrometry (Mosin *et al*., 1996a). The recent advances in technical and computing capabilities of these analytical methods have allowed to considerable increase the efficiency of *de novo* biological studies, as well as to carry out structural-functional biophysical 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 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.

## 2. Material and Methods

### *2.1. Biological Objects*

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.

### *2.2. Chemicals*

For preparation of growth media was used $D_2O$ (99.9 atom.%) and DCl (95.5 atom.%) purchased 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: $Me_4Si$).

### *2.3. Adaptation Technique*

The initial strains were modified by adaptation to deuterium by plating individual colonies onto 2 % (w/v) agarose growth media with stepwise increasing gradient of $D_2O$ concentration and subsequent selection of individual cell colonies stable to the action of $D_2O$. As a source of deuterated growth substrates for the growth of chemoheterotrophic bacteria and chemoorganoheterotrophic 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 of 0.5 N DCl (in $^2H_2O$) and autoclaved for 30–40 min at 0.8 atm. The suspension was neutralized with 0.2 N KOH (in $D_2O$) to pH = 7.0 and used as a source of growth substrates while adaptation and growing of chemoheterotrophic bacterium *B. sublilis* and chemo-organotrophic bacterium *H. halobium*.

### *2.4. Growth Media*

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; $MnSO_4 \cdot 2H_2O$ – $3 \cdot 10^{-4}$; $CaCl_2 \cdot 6H_2O$ – 0.065; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$, $CoCl_2 \cdot 6H_2O$ – $5 \cdot 10^{-6}$.

### *2.5. Growth Conditions*

The cells were grown 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. The bacterial growth was monitored on the ability to form individual colonies on the surface of solid 2 % (w/v) agarose media, as well as on the optical density of the cell suspension measured on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) at $\lambda$ = 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 $^2H_2O$ 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 culture liquid (CL) 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, with using SDS as a detergent (Mosin *et al*., 1999a).

### *2.6. Protein Hydrolysis*

Dry biomass (10 g) was treated with a chloroform–methanol–acetone mixture (2:1:1, % (v/v)) and supplemented with 5 ml 6 N DCl (in $^2H_2O$). The ampoules were kept at 110 $^0$C for ~24 h. Then the reaction mixture was suspended in hot $D_2O$ and filtered. The hydrolysate was evaporated at 10 mm Hg. Residual DCl was removed in an exsiccator over solid NaOH.

### *2.7. Hydrolysis of Intracellular Policarbohydrates*

Dry biomass (50 mg) was placed into a 250 ml round bottomed flask, supplemented with 50 ml distilled $D_2O$ and 1.6 ml 25 % (v/v) $H_2SO_4$ (in $D_2O$), and boiled in a reflux water evaporator for ~90 min. After cooling, the reaction mixture was suspended in one volume of hot distilled $^2H_2O$ and neutralized with 1 N $Ba(OH)_2$ (in $D_2O$) to pH = 7.0. $BaSO_4$ was separated by centrifugation (1500 g, 5 min); the supernatant was decanted and evaporated at 10 mm Hg.

### *2.8. Amino Acid Analysis*

The amino acids of the hydrolyzed biomass were analyzed on a Biotronic LC-5001 (230×3.2 mm) column ("Eppendorf–Nethleler–Hinz", Germany) with a UR-30 sulfonated styrene resin ("Beckman–Spinco", USA) as a stationary phase; the mobile phase: 0.2 N sodium–citrate buffer (pH = 2.5); the granule diameter: 25 μm; working pressure: 50–60 atm; the eluent input rate: 18.5 ml/h; the ninhydrin input rate: 9.25 ml/h; detection at $\lambda$ = 570 and $\lambda$ = 440 nm (for proline).

### *2.9. Analysis of Carbohydrates*

Carbohydrates were analyzed on a Knauer Smartline chromatograph ("Knauer", Germany) equipped with a Gilson pump ("Gilson Inc.", USA) and a Waters K 401 refractometer ("Waters Associates", Germany) using Ultrasorb CN column (250×10 mm) as a stationary phase; the mobile phase: acetonitrile–water (75:25, % (w/w); the granule diameter: 10 μm; the input rate: 0.6 ml/min.

### *2.10. Analysis of Fatty Acids*

Fatty acids were analyzed on a Beckman Gold System (Beckman, USA) chromatograph, equiped with Model 126 UV-Detector (USA). Stationary phase: Ultrasphere ODS, 5 μm, 4.6×250 mm; mobile phase: linear gradient 5 mM $KH_2PO_4$–acetonitrile (shown in phantom), elution rate: 0.5 ml/min, detection at $\lambda$ = 210 nm.

### *2.11. Mass Spectrometry*

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 (Mosin *et al*., 2012). FAB-mass spectra were recorded on a VG-70 SEQ chromatograph ("Fisons VG Analytical", USA) equipped with a cesium $Cs^+$ source on a glycerol matrix with accelerating voltage 5 kV and ion current 0.6–0.8 mA. Calculation of deuterium enrichment of the molecules was carried out using the ratio of contributions of molecular ion peaks of deuterated compounds extracted on $D_2O$-media relative to the control obtained on $H_2O$.

### *2.12. IR-spectroscopy*

IR-spectroscopy was performed on Brucker Vertex spectrometer ("Brucker", Germany) (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 $cm^{-1}$).

### *2.6. Scanning Electrom Microscopy (SEM)*

SEM was carried out on JSM 35 CF (JEOL Ltd., Korea) device, equiped with SE detector, thermomolecular pump, and tungsten electron gun (Harpin type W filament, DC heating); working pressure: $10^{-4}$ Pa ($10^{-6}$ Torr); magnification: 300.000, resolution: 3.0 nm, accelerating voltage: 1–30 kV; sample size: 60–130 mm.

## 3. Results and Discussion

Numerous studies with various biological objects in $D_2O$, carried out by us, proved that when biological objects are being exposed to water with different deuterium content, their reaction varies depending on the isotopic composition of water (the content of deuterium in 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 atoms lies in the range $k_H/k_D$ = 5–8 for C–H versus C–D, N–D versus N–D, and O–H versus O–D-bonds (Mosin, 1996; Mosin & Ignatov, 2012a; Mosin & Ignatov, 2012b). Isotopic effects have an impact not only on the physical and chemical properties of deuterated macromolecules in which H atoms are substituted with D atoms, 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) $D_2O$.

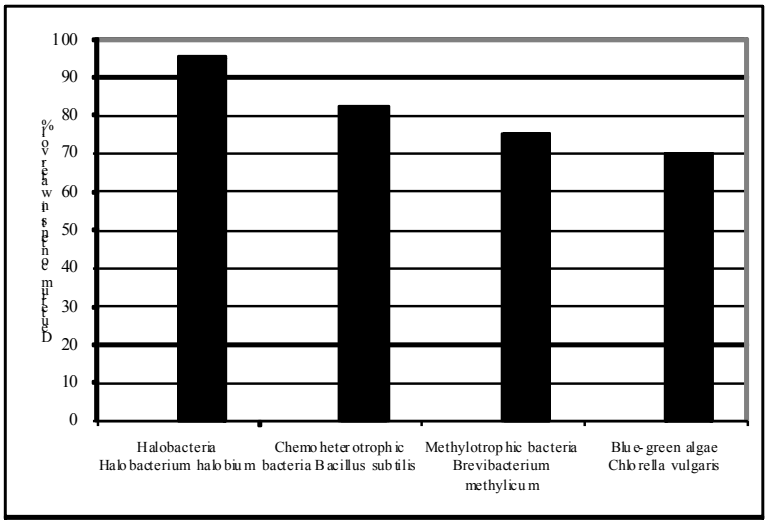



**Fig. 1.** Cell survival of various microorganisms in water with different deuterium content (%, v/v)

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 halobacterium *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 (Mosin & Ignatov, 2014). 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 atom.% D) for biomedical use (Mosin & Ignatov, 2013d), 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 atom.% D) on growth media with high $D_2O$-content (Mosin & Ignatov, 2012b).

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 $D_2O$ retain 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 add of 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 % (v/w) (Mosin *et al*., 1998). 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 (Mosin & Ignatov, 2013e). 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 atom.% $D_2O$ 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 (Mosin & Ignatov, 2013c). The fractionation of inosine from the culture liquid 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 atom.% D) with the inclusion of three deuterium atoms in the ribose and two deuterium atoms in the hypoxanthine fragment of the molecule.

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 97.5 atom.% D. In the case of *C. vulgaris* and *H. halobium* was 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 atom.% $D_2O$ 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) (Mosin & Ignatov, 2014). 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 95.7 atom.% 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 at 70–75 % (v/v) $D_2O$.

The composition of growth media evidently also 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 ~0,2–10 atom.%. 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. The evidence has been obtained that cells evidently are able to regulate the D/H ratios, while its changes trigger distinct molecular processes. One possibility to modify intracellular D/H ratios is the activation of the $H^+$-transport system, which preferentially eliminates $H^+$, resulting in increased D/H ratios within cells (Somlyai *et al*., 2012). Furthermore 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 (Eryomin *et al*., 1978). 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 (Mosin & Ignatov, 2012a; Mosin & Ignatov, 2012b; Mosin & Ignatov, 2014).

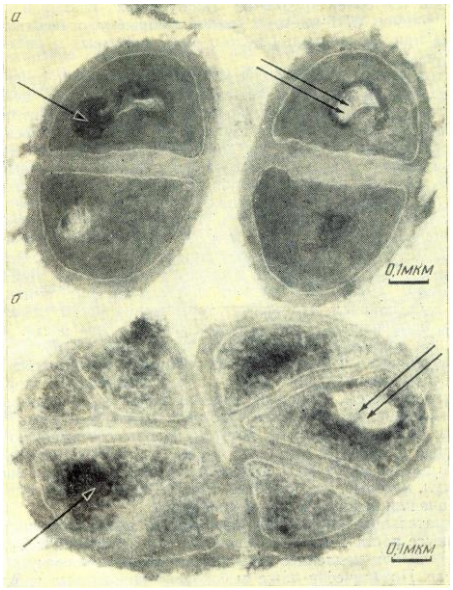



**Fig. 2.** Electron micrographs of *Micrococcus lysodeikticus* cells obtained by SEM method: *a*) – protonated cells obtained on $H_2O$-medium; *b*) – deuterated cells obtained on $D_2O$-medium. The arrows indicate the tightly-packed portions of the membranes

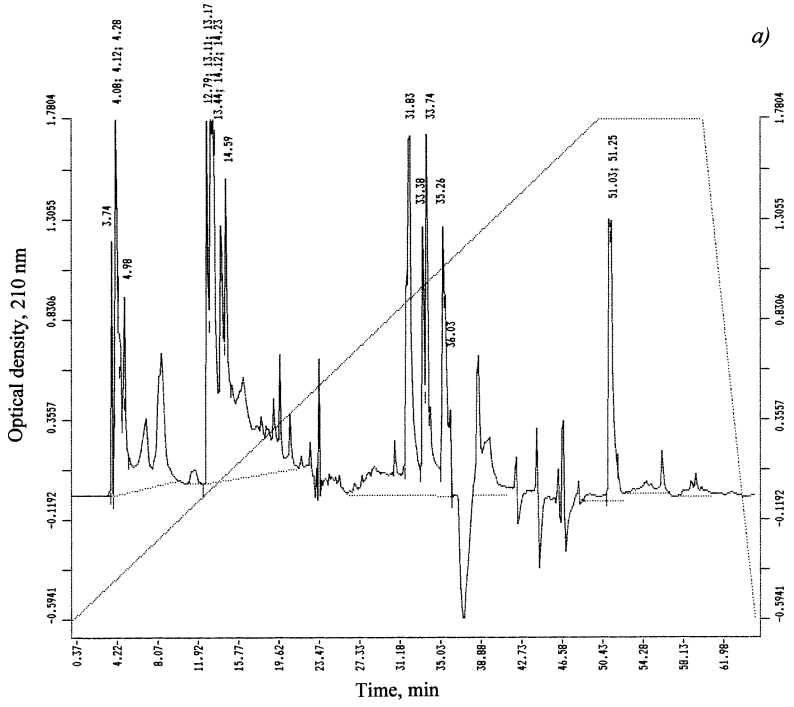
Our data generally confirm a stable notion that adaptation to D2O is a phenotypic phenomenon as the adapted cells eventually return back to the normal growth after some lag-period after their replacement

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 (Mosin et al., 1996b; Mosin & Ignatov, 2012). 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 (Ignatov & Mosin, 2013c)). 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 (Ignatov & Mosin, 2013d). 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 (Cioni & Strambini, 2002).

Evidently the cell implements 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 $H_2O$ (Mosin et al., 1999b). These chemical-physical factors lead to slowing down in the rates of enzymatic reactions in $D_2O$ (Cleland, 1976). 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 during mitosis, leading to permanent changes on DNA structure and consequently on cell genotype (Lamprecht et al., 1989). 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 behavior of an organism in the presence of $D_2O$ (Török et al., 2010). And next, the changes in dissociation constants of DNA and protein ionizable groups when transferring the macromolecule from $H_2O$ into $D_2O$ 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. Hence, the structural and dynamic properties of the cell membrane, which depends on qualitative and quantitative composition of membrane's fatty acids, can also be modified in the presence of $D_2O$. The cellular membrane is one of the most important organelles in the bacteria 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 atom.% $D_2O$, 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 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. 3b). 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.



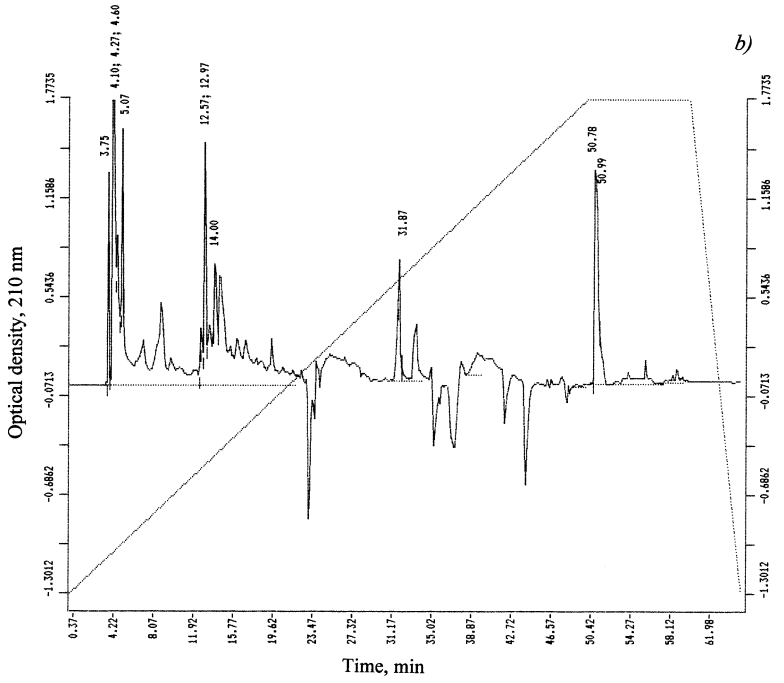



**Fig. 3.** HPLC-chromatograms of fatty acids obtained from protonated (a) and deuterated (b) cells of *B. subtilis* on the maximally deuterated $D_2O$-medium: Beckman Gold System (Beckman, USA) chromatograph (4.6×250 mm); stationary phase: Ultrasphere ODS, 5 μm; mobile phase: linear gradient 5 mM $KH_2PO_4$–acetonitrile (shown in phantom), elution rate: 0.5 ml/min, detection at $\lambda = 210$ nm. The peaks 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 D2O-medium (Fig. 4). Protein hydrolyzates contains fifteen identified amino acids (except proline, which was detected at $\lambda = 440$ 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 atom.% for leucine/isoleucine to 97.5 atom.% for alanine.
Qualitative and quantitative composition of the intracellular carbohydrates of B. subtilis obtained on maximally deuterated D2O-medium is 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) (Fig. 5). 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 H2O except for sucrose in deuterated sample that was not detected (Table 3). The deuterium enrichment levels of carbohydrates were varied from 90.7 atom.% for arabinose to 80.6 atom.% for glucose.

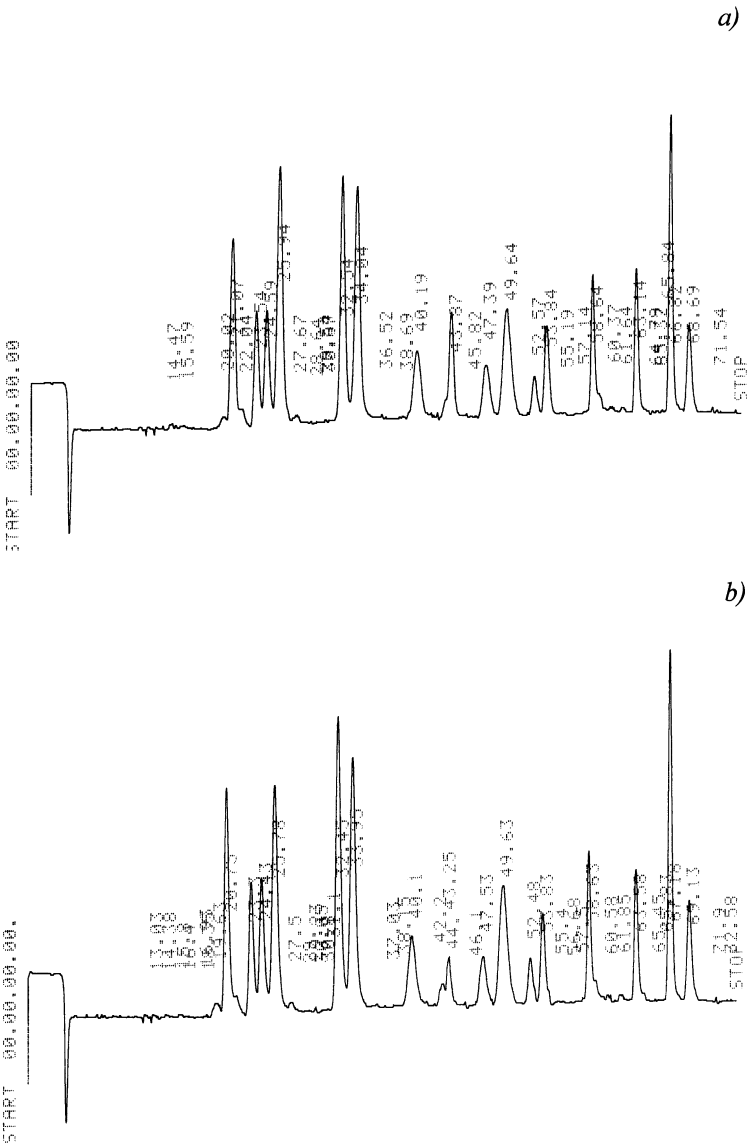



**Fig. 4.** HPLC-chromatograms of amino acids obtained from hydrolysates of protonated (a) and deuterated (b) cells of *B. subtilis* on the maximally deuterated $D_2O$-medium: Biotronic LC-5001 (230×3.2 mm) column ("Eppendorf–Nethleler–Hinz", Germany); stationary phase: UR-30 sulfonated styrene resin ("Beckman–Spinco", USA); 25 μm; 50–60 atm; mobile phase: 0.2 N sodium–citrate buffer (pH = 2.5); the eluent input rate: 18.5 ml/h; the ninhydrin input rate: 9.25 ml/h; detection at $\lambda = 570$ and $\lambda = 440$ nm (for proline).

**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 atom.% $D_2O$ | | |
| 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-5-(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.

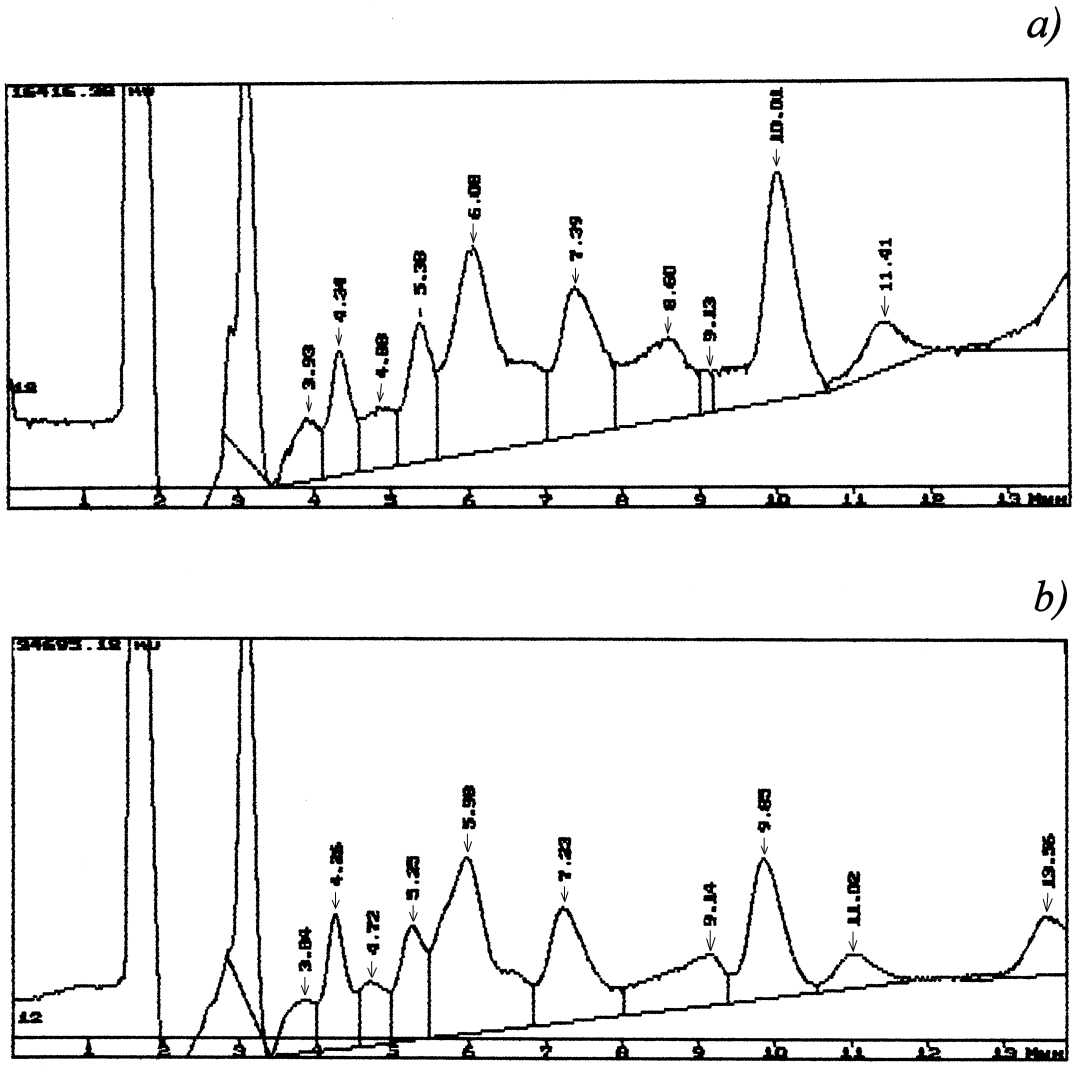



**Fig. 5.** HPLC-chromatograms of intracellular carbohydrates obtained from protonated (a) and deuterated (b) cells of *B. subtilis* on the maximally deuterated $D_2O$-medium: Knauer Smartline chromatograph (250×10 mm) ("Knauer", Germany); stationary phase: Ultrasorb CN; 10 μm; mobile phase: acetonitrile–water (75:25, % (w/w); the input rate: 0.6 ml/min

**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 atom.% $D_2O$** | |
| 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.

In conclusion it should be noted that comparative analysis of IR-spectra of $H_2O$ solutions and its deuterated analogues ($D_2O$, HDO) is of considerable interest because at changing of the atomic mass of hydrogen by deuterium atoms in $H_2O$ molecule their interaction will also change, although the electronic structure of the molecule and its ability to form H-bonds, however, remains the same. The local maximums in IR-spectra reflect vibrational-rotational transitions in the ground electronic state; the substitution with deuterium changes the vibrational-rotational transitions in $H_2O$ molecule, that is why it appear other local maximums in IR-spectra. In the water vapor state, the vibrations involve combinations of symmetric stretch ($\mathbf{v}_1$), asymmetric stretch ($\mathbf{v}_3$) and bending ($\mathbf{v}_2$) of the covalent bonds with absorption intensity ($H_2O$) $\mathbf{v_1; v_2; v_3}$ = 2671; 1178.4; 2787.7 $cm^{-1}$. For liquid water absorption bands are observed in other regions of the IR-spectrum, the most intense of which are located at 2100, $cm^{-1}$ and 710-645 $cm^{-1}$. For $D_2O$ molecule these ratio compiles 2723.7, 1403.5 and 3707.5 $cm^{-1}$, while for HDO molecule – 2671.6, 1178.4 and 2787.7 $cm^{-1}$. HDO (50 mole% $H_2O$ + 50 mole% $D_2O$; ~50 % HDO, ~25 % $H_2O$, ~25 % $D_2O$) has local maxima in IR-spectra at 3415 $cm^{-1}$, 2495 $cm^{-1}$ 1850 $cm^{-1}$ and 1450 $cm^{-1}$ assigned to $OH^-$ -stretch, $OD^-$ -stretch, as well as combination of bending and libration and HDO bending respectively.

In the IR-spectrum of liquid water absorbance band considerably broadened and shifted relative to the corresponding bands in the spectrum of water vapor. Their position depends on the temperature (Ignatov & Mosin, 2013*b*). The temperature dependence of individual spectral bands of liquid water is very complex (Zelsmann, 1995**).** Furthermore, the complexity of the IR-spectrum in the area of $OH^-$ stretching vibration can be explained by the existence of different types of $H_2O$ associations, manifestation of overtones and composite frequencies of $OH^-$ groups in the hydrogen bonds, and the tunneling effect of the proton (for relay mechanism) (Ykhnevitch, 1973). Such complexity makes it difficult to interpret the spectrum and partly explains the discrepancy in the literature available on this subject.

In liquid water and ice the IR-spectra are far more complex than those ones of the vapor due to vibrational overtones and combinations with librations (restricted rotations, i.e. rocking motions). These librations are due to the restrictions imposed by hydrogen bonding (minor $L_1$ band at 395.5 $cm^{-1}$; major $L_2$ band at 686.3 $cm^{-1}$; for liquid water at 0 $^0C$, the absorbance of $L_1$ increasing with increasing temperature, while $L_2$ absorbance decreases but broadens with reduced wave number with increasing temperature (Brubach et al., 2005). The IR spectra of liquid water usually contain three absorbance bands, which can be identified on absorption band of the stretching vibration of $OH^-$ group; absorption band of the first overtone of the bending vibration of the molecule HDO and absorption band of stretching vibration of $OD^-$ group (Max & Chapados, 2009). Hydroxyl group $OH^-$ is able to absorb much infrared radiation in the infrared region of the IR-spectrum. Because of its polarity, these groups typically react with each other or with other polar groups to form intra-and intermolecular hydrogen bonds. The hydroxyl groups, which are not involved in formation of hydrogen bonds usually, produce the narrow bands in IR spectrum, while the associated groups – broad intense absorbance bands at lower frequencies. The magnitude of the frequency shift is determined by the strength of the hydrogen bond. Complication of the IR spectrum in the area of $OH^-$ stretching vibrations can be explained by the existence of different types of associations of $H_2O$ molecules, a manifestation of overtones and combination frequencies of $OH^-$ groups in hydrogen bonding, as well as the proton tunneling effect (on the relay mechanism).

Assignment of main absorbtion bands in the IR-spectrum of liquid water is given in the Table. 4. The IR spectrum of $H_2O$ molecule was examined in detail from the microwave till the middle (4–17500 $cm^{-1}$) visible region and the ultraviolet region – from 200 $nm^{-1}$ to ionization limit at 98 $nm^{-1}$ (Walrafen, 1972). In the middle visible region at 4–7500 $cm^{-1}$ are located rotational spectrum and the bands corresponding to the vibrational-rotational transitions in the ground electronic state. In the ultraviolet region (200 to 98 $nm^{-1}$) are located bands corresponding to transitions from the excited electronic states close to the ionization limit in the electronic ground state. The intermediate region of the IR-spectrum – from 570 nm to 200 nm corresponds to transitions to higher vibrational levels of the ground electronic state.

**Table 4.** The assignment of main frequencies in IR-spectra of liquid water $H_2O$ and $D_2O$

| Main vibrations of liquid $H_2O$ and $D_2O$ | | | | |
|---|---|---|---|---|
| Vibration(s) | $H_2O$ (t = 25 $^0C$) | | $D_2O$ (t = 25 $^0C$) | |
| | v, $cm^{-1}$ | $E_0$, $M^{-1}$ $cm^{-1}$ | v, $cm^{-1}$ | $E_0$, $M^{-1}$ $cm^{-1}$ |
| Spinning $\nu_1$ + deformation $\nu_2$ | 780-1645 | 21.65 | 1210 | 17.10 |
| Composite $\nu_1 + \nu_2$ | 2150 | 3.46 | 1555 | 1.88 |
| Valence symmetrical $\nu_1$, valence asymmetrical $\nu_3$, and overtone $2\nu_2$ | 3290-3450 | 100.65 | 2510 | 69.70 |

At the transition from $H_2O$ monomers to $H_2O$ dimer and $H_3O$ trimer absorption maximum of valent stretching vibrations of the O-H bond is shifted toward lower frequencies ($\mathbf{v}_3$ = 3490 $cm^{-1}$ and $\mathbf{v}_1$ = 3280 $cm^{-1}$) (Eisenberg & Kauzmann, 1969) and the bending frequency increased ($\mathbf{v}_2$ = 1644 $cm^{-1}$) because of hydrogen bonding. The increased strength of hydrogen bonding typically shifts the stretch vibration to lower frequencies (red-shift) with greatly increased intensity in the infrared due to the increased dipoles. In contrast, for the deformation vibrations of the H-O-H, it is observed a shift towards higher frequencies. Absorption bands at 3546 and 3691 $cm^{-1}$ were attributed to the stretching modes of the dimer [$(H_2O)_2$]. These frequencies are significantly lower than the valence modes of $\nu_1$ and $\nu_3$ vibrations of isolated $H_2O$ molecules at 3657 and 3756 $cm^{-1}$ respectively). The absorbtion band at 3250 $cm^{-1}$ represents overtones of deformation vibrations. Among frequencies between 3250 and 3420 $cm^{-1}$ is possible Fermi resonance (this resonance is a single substitution of intensity of one fluctuations by another fluctuation when they accidentally overlap each other). The absorption band at 1620 $cm^{-1}$ is attributed to the deformation mode of the dimer. This frequency is slightly higher than the deformation mode of the isolated $H_2O$ molecule (1596 $cm^{-1}$). A shift of the band of deformation vibration of water in the direction of high frequencies at the transition from a liquid to a solid atate is attributed by the appearance of additional force, preventing O-H bond bending. Deformation absorption band in IR-spectum of water has a frequency at 1645 $cm^{-1}$ and a very weak temperature dependence. It changes little in the transition to the individual $H_2O$ molecule at a frequency of 1595 $cm^{-1}$. This frequency is found to be sufficiently stable, while all other frequencies are greatly affected by temperature changes, the dissolution of the salts and phase transitions. It is believed that the persistence of deformation oscillations is stipulated by processes of intermolecular interactions, i.g. by the change in bond angle as a result of interaction of $H_2O$ molecules with each other, as well as with cations and anions.

Thus the study of the characteristics of the IR spectrum of water allows to answer the question not only on the physical parameters of the molecule and the covalent bonds at isotopic substitution with deuterium, but also to make a certain conclusion on associative environment in water. The latter fact is important in the study of structural and functional properties of water assotiates and its isotopomers at isotopic substitution with deuterium.

**Conclusions**

Our biophysical experiments demonstrated that the effects observed at the cellular growth on $D_2O$ possess a complex multifactor character stipulated by changes of morpholodical, cytological and 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 deuterated 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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