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Research the Influence of Deuterium Depleted and Heavy Types of Water on Biological Objects

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Abstract
This article describes the data on isotopic effects of deuterium in various biological objects as the cells of methylotrophic, chemoheterotrophic, photoorganotrophic microorganisms, green algae as well as animal cells. It was demonstrated that the increased content of deuterium in water leads to physiological, morphological and cytology alterations of the cell, and also renders negative influence on cellular metabolism, while deuterium depleted water (DDW) with decreased deuterium content on 20–30 % exerts beneficial effects on organism. The maximum kinetic isotopic effect measured at ordinary temperatures in chemical reactions leading to rupture of bonds involving hydrogen and deuterium lies in the range $k_H/k_D = 6–8$ for C–H versus C–D, N–D versus N–D, and O–H versus O–D-bonds. By the IR-spectroscopy method water samples with varying content of deuterium were investigated.
Key words: deuterium, heavy water, deuterium depleted water, isotopic effects, biological systems, IR-spectroscopy.

1. Introduction

The most interesting biological phenomenon is the ability of some microorganisms to grow on D_2O media in which all hydrogen atoms are replaced with deuterium (Ignatov & Mosin, 2013a; Ignatov & Mosin, 2013b). The average ratio of H/D in nature makes up approximately 1:5700 (Lis *et al*., 2008). In mixtures D_2O–H_2O with high speed occurs the dissociation reactions and isotopic (H–D) exchange with the formation of semi-heavy water (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 difference in nuclear masses stipulates the isotopic effects, which may be sufficiently essential for H/D pair (Lobishev & Kalinichenko, 1978).

With the development of new biotechnological approaches, there appears an opportunity to use adapted to deuterium cells for preparation of deuterium-labeled natural compounds (Mosin *et al*., 2013a; Mosin *et al*., 2014). The traditional method for production of deuterium labeled compounds consists in the growth on media containing maximal concentrations of D_2O and deuterated substrates as [D]methanol, [D]glucose etc. (Mosin *et al*., 2013b). During the 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 with using a combination of physical-chemical methods of separation – hydrolysis, precipitation and extraction with organic solvents and chromatographic purification by column chromatography on different adsorbents. These deuterium labeled 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 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 possibility 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*., 1996). The recent advances in the technical and computing capabilities of analytical methods have allowed to considerable increase the efficiency of de novo biomedical studies, as well as to carry out structural-functional studies with deuterium labeled molecules on a molecular level.

The aim of this research was to study the isotopic effects of water in biological objects represented by methylotrophic bacteria, chemoheterotrophic bacteria, photo-organotrophic bacteria and microalgae.

2. Material and methods

2.1. The objects of the study

In this study were used various cells of procaryotic and eucaryotic microorganisms, realizing methylotrophic, chemoheterotrophic, photo-organotrophic and photosynthesizing pathways of carbon assimilation.

2.2. Chemical reagents

For preparation of growth media was used D_2O (99.9 atom.%) and DCl (95.5 atom.%) 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 was 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. Isolation of natural compounds

Cellular growth was carried out in 500 ml Erlenmeyer flasks containing 100 ml of growth media with 99.9 atom.% D_2O 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 was carried out at 32 ^{0}C in a photoreactor with CO_2 bubbling. After 6–7 days the cells were harvested and separated by centrifugation (10000 g, 20 min) on T-24 centrifuge ("Heracules Sepatech", Germany). The biomass was washed with D_2O and extracted with a mixture of organic solvents: chloroform-methanol-acetone = 2:1:1, % (v/v) for isolating of 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. Hydrolysis of polysaccharides and amino acids was performed according to standard procedures using as the reactant HCl in D_2O.

2.4. Amino acid analysis

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; 22 ^{0}C; 3.2×230 mm; 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).

2.5. Analysis of carbohydrates

Carbohydrates were analyzed on a Knauer Smartline chromatograph ("Knauer GmbH", Germany) equipped with a Gilson pump ("Gilson Inc.", USA) and a Waters K-401 refractometer ("Waters Corp.", USA) using Separon NH_2 as a stationary phase: 22 ^{0}C; the column size – 4.5 × 250 mm; the granule diameter – 10 μm; the mobile phase – acetonitrile–water (75:25, % (w/w); the input rate – 0.6 ml/min.

2.6. Fatty acid analysis

Fatty acids were analyzed on a Beckman Gold System 166 chromatograph (Beckman Coulter, USA), equipped with Model 166 and UV-detector LCD 2563 (Beckman Coulter, USA). Stationary phase: Ultrasphere ODS, 22 ^{0}C; 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.

2.7. Mass-spectrometry

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. 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 & Ignatov, 2013c). Calculation of levels of deuterium enrichment of molecules was carried by the contribution ratio of the peaks of the molecular ions $[M]^+$ for deuterated derivatives of amino acids, isolated from D_2O-media and protonated amino acids obtained in H_2O-medium (control).

2.8. IR-spectroscopy

IR-spectra of water with varying deuterium content 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}).

3. Results and discussion

3.1. Isotopic effects of deuterium in biological objects

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 measured at ordinary temperatures in chemical reactions leading to rupture of chemical bonds involving hydrogen and deuterium lies in the range k_H/k_D = 6–8 for C–H versus C–D, N–D versus N–D, and O–H versus O–D-bonds.

Water is a solvent in chemical reactions, so at the calculation of isotopic effects of deuterium it is necessary to take into consideration the effect of a solvent. As a universal solvent in which all biological reactions take place, deuterium depleted water increases the rate of these reactions compared with water with natural isotopic composition. This effect is known as kinetic isotopic effect of a solvent.

The natural water consists on 99.7 mol.% of $H_2{}^{16}O$, which molecules are formed by ^1H and ^{16}O atoms. The remaining 0.3 mol.% is represented by isotope varieties (isotopologues) of water molecules, wherein deuterium forms 6 configurations of isotopologues – $HD^{16}O$, $HD^{17}O$, $HD^{18}O$, $D_2{}^{16}O$, $D_2{}^{17}O$, $D_2{}^{18}O$, while 3 configuration are formed by isotopologues of oxygen – $H_2{}^{16}O$, $H_2{}^{17}O$, $H_2{}^{18}O$.

The average ratio of atoms of deuterium and hydrogen in natural waters compiles ~1:5700. 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.

The concentration of water molecules containing heavy isotopes of D, ^{17}O and ^{18}O, in natural water varies within the limits laid down in the basic standards of the isotopic composition of the hydrosphere SNOW and SLAP (Table 1).

According to the international SMOW standard the absolute content of D (isotopic shift, δ, ppm) in sea water: $D/H = (155.76\pm0.05)\cdot10^{-6}$ (155.76 ppm) (Ignatov & Mosin, 2013d). For SLAP standard isotopic shifts for D in seawater: $D/H = 89\cdot10^{-6}$ (89 ppm). Content of the lightest isotopologue – $H_2{}^{16}O$ in water corresponding to SMOW standard is 997.0325 g/kg (99.73 mol.%), and for SLAP standard – 997.3179 g/kg (99.76 mol.%). In surface waters, the ratio $D/H = (1.32–1.51)\cdot10^{-4}$, while in the coastal seawater – $(1.55–1.56)\cdot10^{-4}$.

The natural waters of CIS countries are characterized by negative deviations from SMOW standard to $(1.0–1.5)\cdot10^{-5}$, in some places up to $(6.0–6.7)\cdot10^{-5}$, but there are also observed positive deviations at $2.0\cdot10^{-5}$. Waters of other underground and surface water sources contain varied amounts of deuterium (isotopic shifts) – from δ = +5.0 D,%, SMOW (Mediterranean Sea) up to δ = -105 D,%, SMOW (Volga River).

The equilibrium vapor pressure of water isotopologues is differed quite significantly. The smaller the mass of the water molecule, the higher the vapor pressure, meaning that the vapor being in equilibrium with water, is always enriched with light isotopes of oxygen and hydrogen. Due to relatively low-mass of elements the difference between the mass of isotopes is large, therefore they are fractionated in natural processes: D/H → 100 %, $^{18}O/^{16}O$ → 12.5 %. Isotopes of hydrogen and oxygen are more efficiently fractionated via the processes of evaporation-condensation and water crystallization. The isotopic fractionation is carried out by following methods – isotopic exchange in the presence of Pd and Pt, the electrolysis of water in combination with a catalytic isotopic exchange between H_2O and H_2, column rectification of cooled gaseous H_2, vacuum freezing of cold vapor followed by thawing and other (Mosin, 2012).

Our previous studies demonstrated that D_2O of high concentration is toxic for the organism, chemical reactions are slower in D_2O, compared with ordinary water, the hydrogen bonds formed with participation of deuterium are somewhat more stronger than those ones formed from hydrogen due to the isotopic effect (Mosin & Ignatov, 2012; Mosin & Ignatov, 2014). In mixtures $D_2O–H_2O$ with high rates occurs isotopic (H–D) exchange resulting in formation of semi-heavy water (HDO): $D_2O + H_2O = 2HDO$. 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 bonds formed by deuterium atoms are differed in strength and energy from similar bonds formed by hydrogen. D_2O boils at +101.44 ^{0}C, freezes at +3.82 ^{0}C, has density at 1.1053 g/cm^3 at 20 ^{0}C, and the maximum density occurs not at +4 ^{0}C as in H_2O, but at +11.2 ^{0}C (1.1060 g/cm^3). These effects are reflected in the chemical bond energy, kinetics, and the rate of chemical reactions in D_2O (Mosin, 1996). The chemical reactions and biochemical processes in the presence of D_2O are 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. 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.

Table 1: The calculated mass concentrations of isotopologues in natural water corresponding to international standards of SMOW* and SLAP**

Isotopologue	Molecular mass, u	Isotopic content, g/kg	
		SMOW	SLAP
$^{1}H_2{}^{16}O$	18.01056470	997.032536356	997.317982662
$^{1}HD^{16}O$	19.01684144	0.328000097	0.187668379
$D_2{}^{16}O$	20.02311819	0.000026900	0.000008804
$^{1}H_2{}^{17}O$	19.01478127	0.411509070	0.388988825
$^{1}HD^{17}O$	20.02105801	0.000134998	0.000072993
$D_2{}^{17}O$	21.02733476	0.000000011	0.000000003
$^{1}H_2{}^{18}O$	20.01481037	2.227063738	2.104884332
$^{1}HD^{18}O$	21.02108711	0.000728769	0.000393984
$D_2{}^{18}O$	22.02736386	0.000000059	0.000000018

Notes:

*SMOW (average molecular weight = 18.01528873 u)

**SLAP (average molecular weight = 18.01491202 u)

According to the theory of a chemical bond the breaking up of covalent C–H 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. Thus 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.

Table 2: Changes in the physical properties of water with isotopic substitution by D and ^{18}O

Physical properties	$H_2{}^{16}O$	$D_2{}^{16}O$	$H_2{}^{18}O$
Density at 20 ^{0}C, g/cm^3	0.997	1.105	1.111
Temperature of maximum density, ^{0}C	3.98	11.24	4.30
Melting point under 1 atm, ^{0}C	0	3.81	0.28
Boiling point temperature at 1 atm, ^{0}C	100.00	101.42	100.14
The vapor pressure at 100 ^{0}C, mm Hg	760.00	721.60	758.10
Viscosity at 20 ^{0}C, cP	1.002	1.47	1.056

Animal cells are capable of withstanding up to 25–30 % D_2O, plants – up to 60 % D_2O, whereas cells of protozoa can live up to 90 % D_2O. Deuterium induces physiological, morphological and cytological alterations on the cell on D_2O-media. 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 H_2O, the distribution of DNA in them was non-uniform. The most sensitive to the replacement of H^+ on D^+ are cell systems, using high mobility of protons and high speed of breaking up of hydrogen bonds, as the apparatus of biosynthesis of macromolecules and a respiratory chain. Last fact allows consider the biological effects of D_2O, as complex multifactor influence acting simultaneously on the functional state of a large number of cell systems: metabolism, biosynthesis, transport agents, the structure and function of macromolecules. This results in inhibition of cell growth and cell death followed by gradually increasing the concentration of D_2O in the growth medium. In some cases this occurs even when using aqueous solutions of H_2O with D_2O.

Isotopic effects of deuterium, which occur in macromolecules of even a small difference between hydrogen and deuterium, having the effect upon the structure. The sensitivity of enzyme function to the structure and the sensitivity of nucleic acid function (genetic and mitotic) may lead to a noticeable effect on the metabolic pathways and reproductive behaviour of an organism in the presence of D_2O (Cleland *et al*., 1976). 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 (Cioni & Strambini, 2002). 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 is 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. As a rule, even highly deuterated growth media contain remaining protons ~0.2–10.0 atom.%. These remaining protons facilitate the restructuring to the changed conditions during the adaptation to ^2H_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 $^2H/^1H$ ratios, while its changes trigger distinct molecular processes. One possibility to modify intracellular $^2H/^1H$ ratios is the activation of the H^+-transport system, which preferentially eliminates H^+, resulting in increased $^2H/^1H$ ratios within cells (Somlyai, 2002).

Comparative HPLC method analysis of the fatty acid composition of deuterated cells of the chemoheterotrophic bacterium *B. subtilis*, obtained on the maximum deuterated medium with 99.8 atom.% D_2O, revealed significant quantitative differences in the fatty acid composition compared to the control obtained in ordinary water (Figure 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 (Figure 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, that is why the biosynthesis of fatty acids in the presence of D_2O is inhibited by deuterium, 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 results were observed with the separation of other natural compounds (proteins, amino acids, carbohydrates) extracted from deutero-biomass obtained from maximally deuterated D_2O-medium.

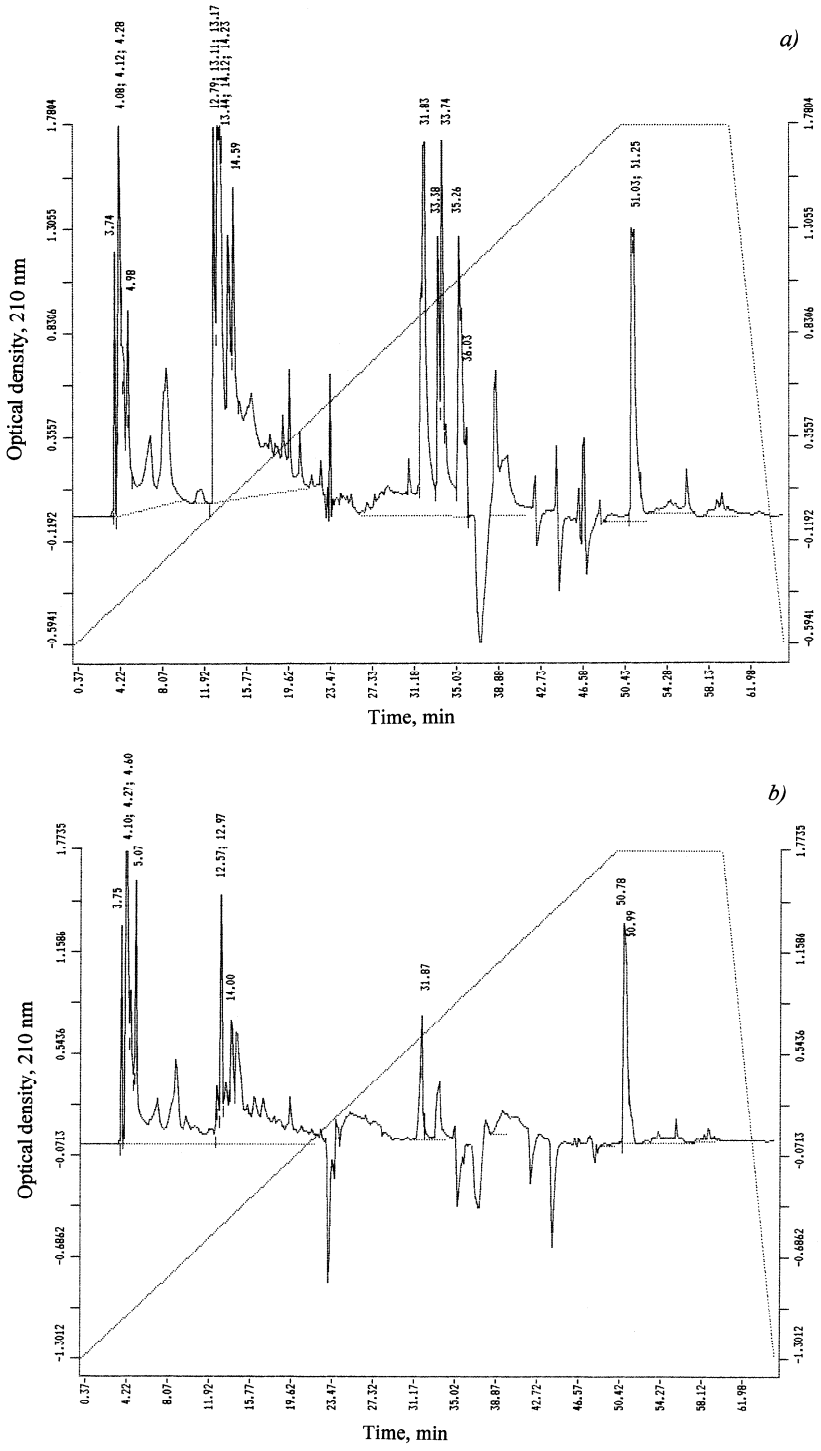

Figure 1. HPLC-chromatograms of fatty acids obtained from protonated (*a*) and deuterated (*b*) cells *B. subtilis* on the maximally deuterated D_2O-medium. The peaks on chromatograms with retention time 3.75 min (instead of 3.74 min in the control), 4.10; 4.27; 4.60 (instead of 4.08; 4.12; 4.28 in the 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

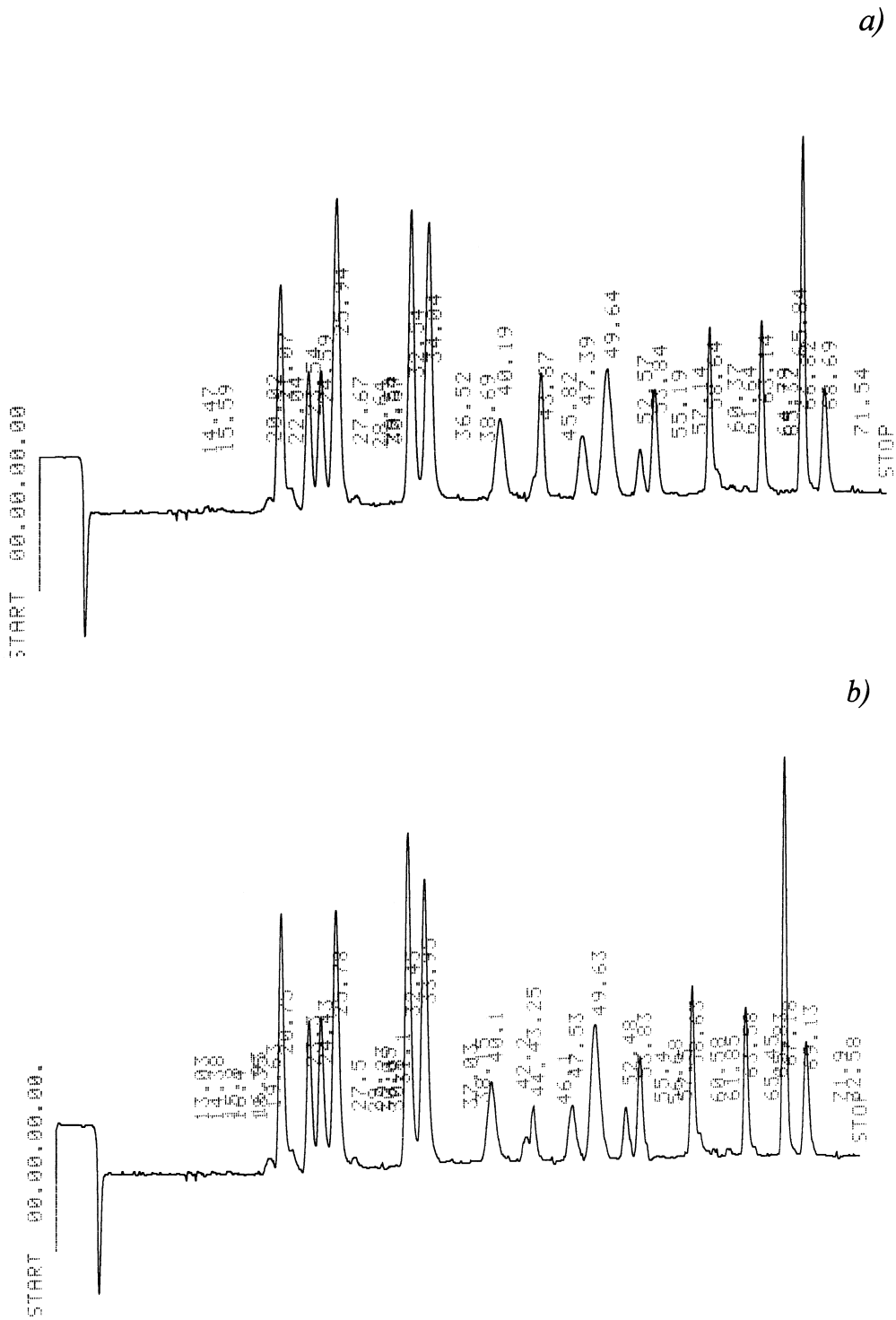

Figure 2. Ion-exchange chromatograms of amino acids obtained from hydrolizates of protonated (a) and deuterated (b) cells of *B. subtilis* on the maximally deuterated D_2O-medium: Biotronic LC-5001 (230×3.2 mm) column ("Eppendorf–Nethleler–Hinz", Germany); stationary phase: UR-30 sulfonated styrene resin ("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 λ = 570 and λ = 440 nm (for proline).

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 (Figure 2). Protein hydrolyzates contains fifteen identified amino acids (except proline, which was detected at λ = 440 nm) (Table 3). 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.o atom.% for leucine/isoleucine to 97.5 atom.% for alanine.

Table 3: 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 99.8 % 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 on growth medium with 99.8 % D_2O and 2 % hydrolysate of deutero-biomass *B. methylicum*.
** 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 Figure 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) (Table 4).
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 the control in H_2O except for sucrose in deuterated sample that was not detected (Table 4).
The deuterium enrichment levels of carbohydrates were varied from 90.7 atom.% for arabinose to 80.6 atom.% for glucose. Thus, the observed changes in the qualitative and quantitative composition of various natural compounds synthesized in the presence of D_2O is a common phenomenon observed for the studied microbial cells.

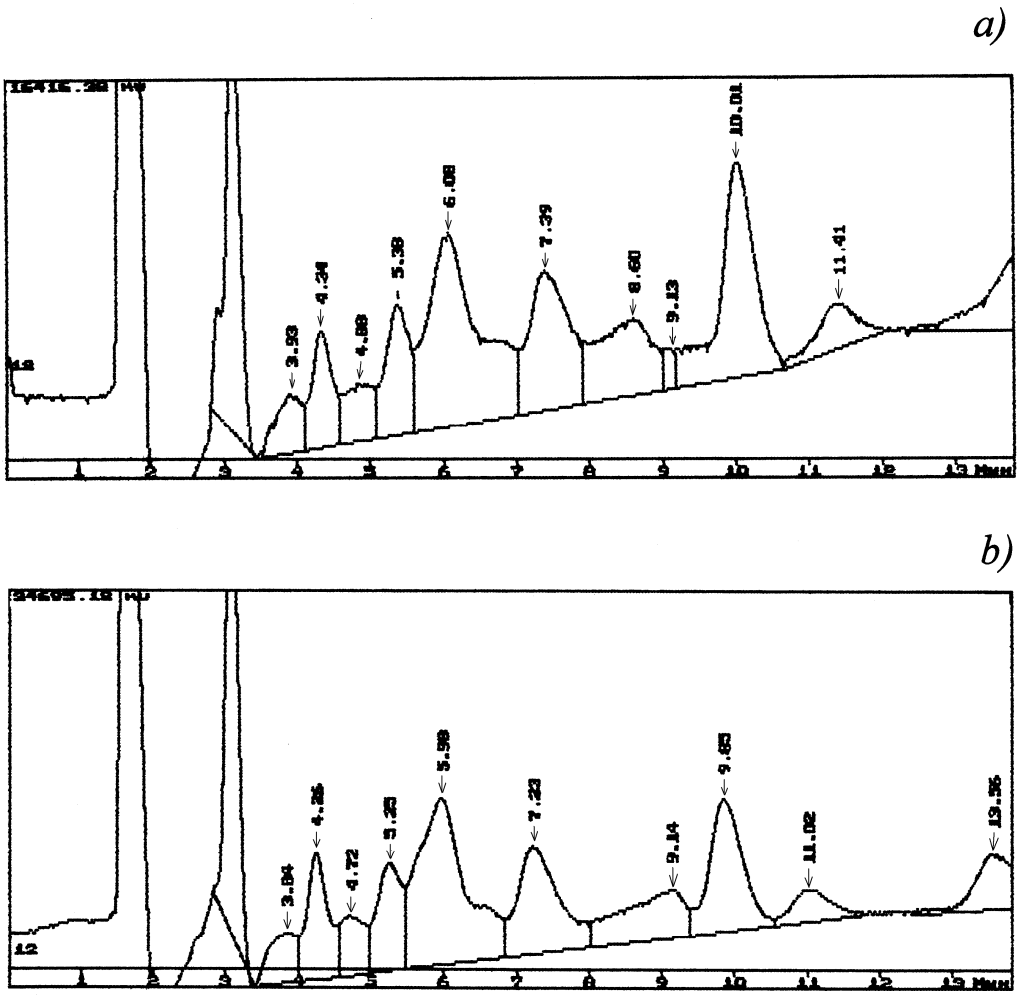

Figure 3. HPLC-chromatograms of intracellular carbohydrates obtained from protonated (a) and deuterated (b) cells of *B. subtilis* on the maximally deuterated D_2O-medium: Knauer Smartline chromatograph (250×10 mm) ("Knauer", Germany); stationary phase: Ultrasorb CN; 10 μm; mobile phase: acetonitrile–water (75:25, % (w/w); the input rate: 0.6 ml/min

Table 4: 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 99.8 % 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	not detected	–

Notes:

* The data were obtained on growth medium with 99.8 % D_2O and 2 % hydrolysate of deutero-biomass *B. methylicum*.

3.2. Biological effects of DDW

Contrary to D_2O, water with the reduced deuterium content (deuterium depleted water, DDW) exerts a positive effect on metabolism. Experiments on animals (Bad'in & Gasteva, 2004) demonstrated that at the consumption of water with decreased content of deuterium pigs, rats and mice provide a larger number of offspring, upkeep of poultry from 6 day old to puberty on DDW leads to the accelerated development of reproductive organs (size and weight) and strengthen the process of spermatogenesis, egg laying by hens is increased by almost half, wheat ripens earlier and gives higher yields. DDW delays the appearance of the first metastasis nodules on the spot of inoculation of cervical cancer, and exerts immunomodulatory and radioprotective effect (Rakov, 2007).

Radioprotective effects of DDW were studied by W. Bild *(*Bild *et al*., 1999*),* V.S. Turusov (Turusov *et al*., 2005) and D.V. Rakov (Rakov *et al*., 2006) at irradiation of mice's cells by γ-radiation at semimortal dose LD50. Survival level of animals treated with deuterium depleted water for 15 days prior to γ-radiation, was 2.5-fold higher than in control group (dose of 850 R). The surviving experimental group of mice has the number of leukocytes and erythrocytes in the blood remained within the normal range, while in the control group the number of leukocytes and erythrocytes was significantly decreased.

Consumption of DDW by cancer patients during or after radiation therapy treatments allows restore the composition of blood and relieve nausea (Olariu *et al*., 2010). According to G. Shomlai, the results of clinical trials with DDW conducted in 1998–2010 in Hungary showed that the survival rate for patients drinking DDW in combination with traditional therapies was significantly higher than for patients who only were treated with chemotherapy or radiation therapy (Somlyai, 2001).

Biological experiments with DDW carried out in Moscow Research Oncological Institute after P.A. Herzen and N.N. Blokhin with Institute of Biomedical Problems (Sinyak *et al*., 1998; Grigoriev *et al*., 2005), confirmed the inhibitory effects of deuterium depleted water on the process of growth of various tumors, i.g. division of breast adenocarcinoma MCF-7 tumor cells being placed in deuterium depleted water started with a delay of ~5–10 hours. In 60 % of mice with immunosuppressed immunity and transplanted human breast tumor MDA and MCF-7 consumption of deuterium depleted water caused tumor regression. A group of mice with transplanted human prostate tumor PC-3 consumed deuterium depleted water showed the increase in the survival rate by ~40 %; the ratio number of dividing cells in tumors of dead animals in experimental group was 1.5:3.0, and in control group – 3.6:1.0 (Turusov *et al*., 2006). In this regard special attention deserves two indicators: the delay of metastasis and loss of animal's weight during experiments. Stimulating action of deuterium depleted water on the immune system of animals has led to delay of development of metastasis by 40 % in comparison with the control group, and weight loss in animals that consumed deuterium depleted water at the end of the experiment was 2 times less. It was also reported that deuterium depleted water may delay the progression of prostate cancer (Kovács *et al*., 2011) and inhibit human lung carcinoma cell growth by apoptosis (Cong *et al*., 2010) – the programmed cell death, resulting in fragmentation of the cell into separate apoptotic bodies bounded by the plasma membrane.

Preliminary experimental results on motility of human sperm (Lobyshev & Kirkina, 2012), indicated that in DDW (4 ppm) spermatozoa longer retain their functional activity, and it increases with a decrease in the deuterium content of water, whereas the sperm motility is by 40 % higher for 5 hours after registration. However, the effect depends on initial properties of a sperm sample. These data indicated that deuterium content variation in water including deep deuterium depletion produces various non-linear isotopic effects on key processes in the cell: enzyme action of Na, K-ATPase, regeneration, motility, fertilizing effectiveness and embryo developing. It should be noted that for any deuterium concentration dependence there should be an optimal condition for the best result.

One prominent effect of deuterium depletion is the inhibition of fatty acids as well as the synthesis, chain elongation and desaturation. These anabolic reactions utilize acetyl-CoA, as well as hydrogen of water for new fatty acid biosynthesis (Boros & Somlyai, 2012). Fatty acids then are used for new membrane formation in the rapidly proliferating cell. The complex structure and molecular organization of the mammalian fatty acid synthase (EC 2.3.1) offer remarkable opportunities with altered morphology and flux handling properties.

The positive influence of drinking deuterium depleted water on blood chemistry included a significant reduction of glucose, cholesterol, erythrocyte sedimentation rates, leukocyte counts and cortisol (stress hormone) levels, while also revealed an increase in antioxidant capacities [Andreeva *et al*., 2005; Burdeynaya *et al*., 2012; Olariu *et al*., 2010). These data evidence the significance of deuterium depleted water to increase energy resources even in a healthy cohort, while decreasing risks of psycho-emotional stress, which is known to pose a negative influence on blood biochemistries that often lead to psychosomatic diseases and shorten life. It was also noted the positive impact of water on indicators of saturation the liver tissue by oxygen: the observed increase in pO_2 was ~15 %, i.e., cell respiration increased 1.3 times (Kolesov & Pomytkin, 2006). On beneficial effect on health of experimental mice evidenced the increased resistance and weight increase compared with the control group.

The main impact of DDW on organism is explained by a gradual reduction of deuterium content in physiological fluids due to the reactions of isotopic (H–D) exchange: $D_2O + H_2O = 2HDO$. It was recorded the change in the

isotopic composition of urine and Ca^{2+} content as well. Thus, the regular consumption of DDW provides a natural way to reduce the deuterium content in the human body to a value of 110 ppm.
Clinical trials of DDW with a residual content of deuterium 60–100 ppm, showed (Turova, 2003) that it can be recommended as an adjunct in the treatment of patients having metabolic syndrome (hypertension, obesity, impaired glucose metabolism) and diabetes. In addition DDW improves the quality of life for patients having renal stone disease (nephrolithiasis) and various disorders in the gastrointestinal tract (colitis and gastritis), cleanses the body of toxins, enhances the action of drugs and promotes weight correction. DDW can be recommended for fast and deep cleaning of the human body from deuterium that is essential for metabolic disturbances. Taking into consideration the dynamics of the distribution of water in the human body, the reaction of isotopic (H/D and $^{16}O/^{18}O$) exchange and the results obtained with DDW, it can be expected that the greatest effect the isotopic purification of water will have on the regulatory system and metabolism.

Table 5: Gradual decreasing of deuterium content in the human body over time, with regular consumption of DDW*

Number of days	The residual content of deuterium in water, ppm		
	60	100	100
	Daily consumption of DDW, liters		
0	1	1	1.5
1	150.5	150.7	150.8
2	145.5	147.9	146.9
7	136.5	143.6	140.5
14	130.6	138.3	134.7
21	120.8	135.6	129.6
28	120.0	133.9	126.6
35	119.6	132.6	124.5
45	117.3	131.5	122.6

*Notes:
The calculation was performed based on the following data:
- daily consumption of DDW – 1 or 1.5 liter;
- daily water exchange rate – 2.5 liters;
- deuterium content in the body corresponds to its content in natural water ~ 150 ppm;
- the average volume of water in the body – 45 liters (average body weight ~ 75 kg).

The total effects of DDW depend on total body mass, total mass of intracellular water, the amount of daily consumption of DDW and the degree of its isotope purification (Ignatov & Mosin, 2014b). The results on the calculation of gradual increasing of deuterium content in the human body at regular consumption of DDW with varied residual deuterium content are shown in Table 5. These results demonstrate that the content of deuterium in the human body decreases while consuming DDW. Thus, at the consumption of water with a residual deuterium content of 60 ppm deuterium content in the body decreases after 45 days to 117.3 ppm, and at the consumption of water with a residual content of deuterium 100 ppm – to 131 ppm at 1 liter of water consumption per a day, to 122.6 ppm at water consumption of 1.5 liters of water a day. Hence, the regular taking of DDW provides a natural way to reduce the content of HDO in the human body to a value of ~117 ppm.

3.4. IR-spectroscopy of D_2O-solutions.

The comparative analysis of IR-spectra of H_2O solutions and its deuterated analogues (D_2O, HDO) is of considerable interest for biophysical studies, 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 remains the same. The IR spectra of water usually contain three absorption bands, which can be identified as 1 – absorption band of the stretching vibration of OH^- group; 2 – absorption band of the first overtone of the bending vibration of the molecule HDO; 3 – absorption band of stretching vibration of OD^- group. OH^- group 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 not involved in formation of hydrogen bonds are usually given the narrow bands in IR spectrum and the associated groups – broad intense absorption 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, a manifestation of overtones and combination frequencies of OH^- groups in

hydrogen bonding, as well as the proton tunneling effect (on the relay mechanism. Such complexity makes it difficult to interpret the IR spectrum and partly explains the discrepancy in the literature available on this subject.

The local maximums in IR-spectra reflect vibrational-rotational transitions in the ground electronic state; the substitution with deuterium changes the vibration-rotational transitions in H_2O molecule that is why there appear other local maximums in IR-spectra. In the water vapor state, the vibrations involve combinations of symmetric stretch (v_1), asymmetric stretch (v_3) and bending (v_2) of the covalent bonds with absorption intensity (H_2O) $v_1;v_2;v_3$ = 2671; 1178.4; 2787.7 cm^{-1}. For liquid water absorption the 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% ^2H_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, 2013e). The temperature dependence of individual spectral bands of liquid water is very complex. 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). 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 vibration overtones and combinations with librations (restricted rotations, e.g. 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 absorption bands in the IR-spectrum of liquid water is given in 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 vibration-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 vibration levels of the ground electronic state.

Results of IR-spectroscopy with device Infra Spec VFA-IR show that at 4.1 μm, even at low concentrations of deuterium of 0.35 and 0.71 %, there is observed a decline in the local maximums relative to the local maximum of 100 % pure water (the local maximums in IR-spectra reflect vibration-rotational transitions in the ground electronic state because at changing the atomic mass of hydrogen and deuterium atoms in the water molecule their interaction will also change, although the electronic structure of the molecule and its ability to form H-bonds, however, remains the same; with the substitution with deuterium the vibration-rotational transitions are changed, that is why it appears other local maximums in IR-spectra. These data are shown in Figure 4. The result is reliable regarding the content of deuterium in natural waters at 0.015–0.03 %.

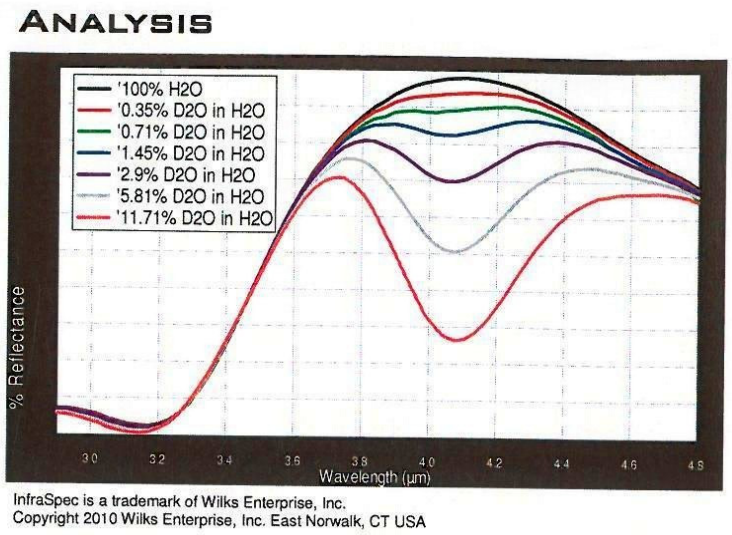

Figure 4: The typical IR-spectra of water with varying content of deuterium

At further transition from H_2O monomers to H_4O_2 dimmer and H_6O_3 trimmer absorption maximum of valent stretching vibrations of the O-H bond is shifted toward lower frequencies (ν_3 = 3490 cm^{-1} and ν_1 = 3280 cm^{-1}) and the bending frequency increased (ν_2 = 1644 cm^{-1}) because of hydrogen bonding (Zelsmann, 1995). The increased strength of hydrogen bonding typically shifts the stretch vibration to lower frequencies (red-shift) with greatly increased intensity in the infrared region 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 dimmer [$(H_2O)_2$]. These frequencies are significantly lower than the valence modes of ν_1 and ν_3 vibrations of isolated H_2O molecules at 3657 and 3756 cm^{-1} respectively). The absorption 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 fluctuation by another fluctuation when they accidentally overlap each other). The absorption band at 1620 cm^{-1} is attributed to the deformation mode of the dimmer. 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 state is attributed by the appearance of additional force, preventing O-H bond bending. Deformation absorption band in IR-spectrum of water has a frequency at 1645 cm^{-1} and 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, e.g. by the change in bond angle as a result of interaction of H_2O molecules with each other, as well as with various cations and anions.

Table 6: The assignment of main frequencies in IR-spectra of liquid H_2O and D_2O

Main vibrations of liquid H_2O and ^2H_2O				
Vibration(s)	H_2O (t = 25 ^{0}C)		D_2O (t = 25 ^{0}C)	
	ν, cm^{-1}	E_0, M^{-1} cm^{-1}	ν, cm^{-1}	E_0, M^{-1} cm^{-1}
Spinning ν_1 + deformation ν_2	780–1645	21.65	1210	17.10
Composite $\nu_1 + \nu_2$	2150	3.46	1555	1.88
Valence symmetrical ν_1, valence asymmetrical ν_3, and overtone $2\nu_2$	3290–3450	100.65	2510	69.70

Thus the study of the characteristics of IR spectra 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 associates and its isotopologues at the isotopic substitution with deuterium.

4. Conclusion

Isotopic effects are determined by an increase in the nuclear mass of deuterium relative to hydrogen, which may be sufficiently essential for the $^{1}H/^{2}H$ pair. The maximum kinetic isotopic effect measured at ordinary temperatures in chemical reactions leading to rupture of bonds involving hydrogen and deuterium lies in the range $k_H/k_D = 6–8$ for C–H versus C–D, N–D versus N–D, and O–H versus O–D-bonds. D_2O of high content of deuterium (99.8 atom.%) leads to physiological, morphological and cytology alterations of the cell, and also renders negative influence on cellular metabolism, while DDW with decreased deuterium content on 20–30 % exerts beneficial effects on metabolism. 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 which use high mobility of protons and high speed of breaking up of hydrogen bonds.

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