

Research of Isotopic Effects in Deuterium in Cells of Microorganisms in the Presence of D₂O and IR spectra in Hot Mineral Water for Origin of Life

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Abstract. In the present paper the isotopic composition of water and its temperature is analyzed. It was proposed an assumption, that under conditions of the primary O₂ free atmosphere, under influence of short-wave solar radiation, geothermal energy and powerful spark discharges, HDO could be collected in hydrosphere, which physical-chemical properties differ from those of H₂O. It were obtained adapted to the maximal concentration D₂O cells of various microorganisms realizing methylotrophic, chemoheterotrophic, photoorganotrophic, and photosynthetic pathways of assimilation of carbon substrata, all biological material of which instead of hydrogen contains deuterium. Their studying will allow to give the answer as to function of deuterated macromolecules in conditions of primary hydrosphere and hot D₂O-solutions. Also were performed experiments for the research of hot mineral, sea and mountain water from Bulgaria with IR spectroscopy.

Key words: deuterium, heavy water, hot mineral water, hydrosphere, evolution

1. Introduction

Natural prevalence of deuterium makes up approximately 0,015 at. % D, and depends strongly on the uniformity of substance and the total amount of matter formed in the course of early evolution. Constant sources of deuterium are explosions of nova stars and thermonuclear processes occurring inside the stars. Probably, it could explain a well known fact why the amounts of deuterium are increased slightly during the global changes of climate in warming conditions. Gravitational field of the Earth is insufficiently strong for retaining of lighter hydrogen, and our planet is gradually losing hydrogen as a result of its dissociation into interplanetary space. Hydrogen evaporates faster than heavy deuterium which is capable to be collected by the hydrosphere. Therefore, as a result of this natural process of fractionation of isotopes H/D throughout the process of Earth evolution there should be an accumulation of deuterium in hydrosphere and surface waters, while in atmosphere and in water vapor deuterium contents are lower. Thus, on the planet there is

going on a natural process of separation of H and D isotopes, playing an essential role in maintenance of life on the planet.

The absolute contents of deuterium (isotopic shifts, δ , ppm) according to the international standard VSMOW, corresponding to Pacific ocean water which is rather stable on isotopic composition, compile $D/H = (155,76 \pm 0,05) \cdot 10^{-6}$ (155,76 ppm). For the international standard SLAP of natural water of Antarctic Region containing less deuterium, the absolute contents of deuterium compile $D/H = 89 \cdot 10^{-6}$ (89 ppm). The average ratio of H/D in nature compiles 1 : 5700. In natural waters the contents of deuterium are distributed non-uniformly: from 0,015 at.% D for water from the Antarctic ice - the most deuterium depleted natural water with deuterium contents in 1,5 times smaller, than in sea water, up to 0,02-0,03 at.% D for river and sea water. Thawed snow and glacial waters in mountains and some other regions of the Earth usually contain on 3-5% less deuterium, than drinking water. On the average, 1 ton of river water contains approximately 150-300 g of deuterium. Other natural waters contain varying levels of deuterium from $\delta = +5,0$ D, %, SMOW (Mediterranean Sea) up to $\delta = -105$ D, %, SMOW (Volga River).

The proposition was made that primary water could contain more deuterium in early stages of evolution, and deuterium was distributed non-uniformly in hydrosphere and atmosphere. As is known, the primary reductive atmosphere of the Earth, consisted basically of gas mixture CO, H₂, N₂, NH₃, CH₄, was lacked O₂-O₃ layer protecting the Earth surface from rigid short-wave solar radiation carrying huge energy capable to cause photolysis and radiolysis of water. The processes accompanying accumulation of deuterium in hydrosphere were solar radiation, volcanic geothermal processes and electric categories in electric discharges in atmosphere. These natural processes could lead to enrichment of hydrosphere by deuterium in the form of HDO which evaporates more slowly than H₂O, and condenses faster. The formation of HDO occurs in D₂O-H₂O mixtures via isotopic exchange: $H_2O + D_2O = 2HDO$, causing deuterium at small amounts to be present in water in form of HDO, and at high amounts - in form of D₂O. The structure of molecules D₂O is the same, as that of H₂O, with very small distinction in values of lengths of covalent bonds. D₂O boils at 101,44 °C, freezes at 3,82 °C, has density at 20 °C 1,105 g/cm³, and the maximum of density is not on 4 °C, as for usual water, but on 11,2 °C (1,106 g/cm³). These effects are reflected in energy of a chemical bond, kinetics and chemical reactions rates in D₂O-H₂O mixtures. Enzymic reactions in D₂O are considerably slowed down. However, there are also such reactions which rates in D₂O are higher, than in H₂O. Basically, they are reactions catalyzing by ions D₃O⁺ or H₃O⁺ or OD⁻ and OH⁻. According to the theory of chemical bond, breaking up of H-O bonds can occur faster, than D-O bonds, mobility of ion D₃O⁺ is lower on 28,5 % than H₃O⁺ ion, and OD⁻ ion is lower on 39,8 % than OH⁻ ion, the constant of ionization of D₂O is less than constant of ionization of H₂O. The maximum kinetic isotopic effect at ordinary temperatures in a chemical reaction leading to rupture of bonds involving H and D was calculated, and the maximum ratio k_H/k_D in macromolecules is in the range of 6 to 8 for C-H versus C-D, N-H versus N-D, and O-H versus O-D bonds.

Deuterated cells of various microorganisms adapted to the maximal concentration of D₂O in growth media (95-98 vol.% D) are convenient objects for evolutionary and adaptation studies as well as structural-functional studies. During the cellular growth on D₂O media there are synthesized macromolecules in which hydrogen atoms in carbon skeletons are almost completely replaced on deuterium. Such deuterated macromolecules undergo the structural-adaptive modification changes necessary for

normal functioning of cells in the presence of D₂O.

Practical interest to further applying of deuterated cells of various microorganisms in researches on their basis mechanisms of cellular adaptation to D₂O and molecular evolution, has predetermined a direction of our studies. The purpose of the present research was studying of isotope effects of deuterium and conditions of primary hydrosphere (temperature, value pH, isotopic composition). In frames of the research were studied various samples of water from Bulgaria.

2. Materials and methods

Researches were carried out with using of microorganisms, realizing methylotrophic (obligate and facultative methylotrophic bacteria *Brevibacterium methylicum* and *Methylobacillus flagellatum*), chemoheterotrophic (*Bacillus subtilis*), photoorganoheterotrophic (Halobacterium *Halobacterium Halobium*) and photosynthetic (blue-green algae *Chlorella vulgaris*) ways of assimilation of substrata.

Samples of water for the research by the IR-spectroscopy method were taken from various sources of Bulgaria: 1 – cactus juice; 2 – hot mineral water (Rupite, Bulgaria); 3 – sea water (a resort Varna, Bulgaria); 4 – mountain water (Teteven, Bulgaria); 5 – deionized water.

Also cactus juice of *Echinopsis pachanoi* was investigated by the IR-spectroscopy method.

For preparation of growth media it was used D₂O (99,8 at. % D), DCl (95,5 at. % D) and [D]methanol (97,5 at. % D), received from the Russian research centre "Isotope" (St. Petersburg, the Russian Federation). Inorganic salts and glucose were preliminary crystallized in D₂O and dried in vacuum before using. D₂O distilled over KMnO₄ with the subsequent control of isotope enrichment by ¹H-NMR-spectroscopy on device Bruker WM-250 ("Bruker", Germany) (working frequency 70 MHz, internal standard Me₄Si).

For cell cultivation and adaptation studies were used growth media with an increasing gradient of ²H₂O concentration from 0; 24,5; 49,0; 73,5 up to 98 vol.% ²H₂O. Cultivation of methylotrophic and chemoheterotrophic bacteria was carried out on minimal salt medium M9 (g/l) KH₂PO₄ – 3; Na₂HPO₄ – 6; NaCl – 0,5; NH₄Cl – 1 with 1–2 vol.% [²H]methanol. Cultivation of chemoheterotrophic bacteria was carried out on FM medium (m/m.%): glucose – 12; yeast extract – 2,5; NH₄NO₃ – 3; MgSO₄·7H₂O – 2; chalk – 2. Cultivation of photoorganoheterotrophic bacteria was carried out on TS medium (g/l): (*D,L*-Ala – 0,43; *L*-Arg – 0,4; *D,L*-Asp – 0,45; *L*-Cys – 0,05; *L*-Glu – 1,3; *L*-Gly – 0,06; *D,L*-His – 0,3; *DL*-Ileu – 0,44; *L*-Leu – 0,8; *L*-Lys – 0,85; *D,L*-Met – 0,37; *D,L*-Phe – 0,26; *L*-Pro – 0,05; *D,L*-Ser – 0,61; *D,L*-Thr – 0,5; *L*-Tyr – 0,2; *D,L*-Trp – 0,5; *D,L*-Val – 1,0; AMF – 0,1; UMF – 0,1; NaCl – 250; MgSO₄·7H₂O – 20; KCl – 2; NH₄Cl – 0,5; KNO₃ – 0,1; KH₂PO₄ – 0,05; K₂HPO₄ – 0,05; Na⁺-cytrate – 0,5; MnSO₄·2H₂O – 3·10⁻⁴; CaCl₂·6H₂O – 0,065; ZnSO₄·7H₂O – 4·10⁻⁵; FeSO₄·7H₂O – 5·10⁻⁴; CuSO₄·5H₂O – 5·10⁻⁵; glycerine – 1,0; (biotin – 1·10⁻⁴; folic acid – 1,5·10⁻⁴; vitamine B₁₂ – 2·10⁻⁵). Blue-green algae grew on Tamia growt medium (g/l): KNO₃ – 5,0; MgSO₄·7H₂O – 2,5; KH₂PO₄ – 1,25; FeSO₄ – 0,003; MnSO₄·2H₂O – 3·10⁻⁴; CaCl₂·6H₂O – 0,065; ZnSO₄·7H₂O – 4·10⁻⁵; CuSO₄·5H₂O – 5·10⁻⁵; CoCl₂·6H₂O – 5·10⁻⁶.

For adaptation were used solid 2% agarose media M9 with gradually increasing concentrations of ²H₂O, combined with the subsequent selection of clones resistant to deuterium.

The cells were grown in 250 ml Erlenmeyer flasks containing 20 ml of the medium at 32-34 °C and vigorously aerated on an orbital shaker Biorad ("Biorad Labs", Poland). Photoorganoheterotrophic bacteria and blue-green algae were grown up at 38 °C at illumination by fluorescent lamps. Bacterial growth was

defined on ability to formation of separate colonies on a surface of 2% agarose media, and on absorbance of cell suspension measured on spectrophotometer Beckman DU-6 (Beckman Coulter, USA) at λ 620 nm.

The analysis of protein hydrolysates was carried out using Biotronic LC 50001 chromatograph ("Eppendorf-Nethleler-Hinz", Germany), 230 x 3.2 mm, working pressure 50-60 atm, flow-rate 18,5 ml/h.

The levels of deuterium enrichment were defined on pulse mass spectrometer VG-70 SEQ ("Fisons VG Analytical", the USA), supplied with caesium source Cs^+ on a glyceric matrix with accelerating pressure 5 kV and an ionic current 0,6–0,8 mA and 1H -NMR-spectroscopy on device Bruker WM-250 ("Bruker", Germany) (working frequency of 70 MHz, internal standard Me_4Si).

IR-spectra of water samples were registered on Fourier-IR spectrometer Bruker Vertex ("Bruker", Germany) (a spectral range: average IR – 370–7800 cm^{-1} ; visible – 2500–8000 cm^{-1} ; the permission – 0,5 cm^{-1} ; accuracy of wave number – 0,1 cm^{-1} on 2000 cm^{-1}). Thermo Nicolet Avatar 360 Fourier-transform IR (M. Chakarova); Differential Non-equilibrium Spectrum (DNES).

3. Results and discussion

We have investigated isotopic effects of deuterium in prokaryotic and eukaryotic cells of various taxonomic groups of microorganisms realizing methylotrophic, hemoheterotrophic, photoorganotrophic and photosynthetic ways of assimilation of carbon substrates (methylotrophic bacteria, halobacteria, blue-green algae) in D_2O with using 1H -NMR-, IR-, and mass-spectrometry technique. The method of step by step adaptation is developed for adaptation of cells of various microorganisms to D_2O consisting in plating initial cells on firm (2% agarose) growth media with increasing gradient of D_2O concentration (from 0; 24,5; 49,0; 73,5 to 98 % D_2O) and the subsequent selection of clones resistant to deuterium. Cells grown on media with a low gradient of D_2O concentration were transferred on media with big gradient of concentration, up to 98 % D_2O . Degree of cell survive on maximum deuterated media was about 40%.

Our experiments demonstrated, that the effects observed at the cellular growth on D_2O possess complex multifactorial character connected to changes of morphological, cytological and physiological parameters – magnitude of the log-period, time of cellular generation, outputs of biomass, a ratio of amino acids, protein, carbohydrates and lipids synthesized in D_2O , and with an evolutionary level of organization of investigated object as well. The general feature of bacterial growth in D_2O was the proportional increase in duration of the log-period and time of cellular generation at reduction of outputs of a microbic biomass. The experimental data testify that cells realize 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 consider adaptation to D_2O as adaptation to the nonspecific factor effecting simultaneously functional condition of several numbers of cellular systems: metabolism, ways of assimilation of carbon substrates, biosynthetic processes, and transport function, structure and functions of macromolecules. There is evidence that during adaptation to D_2O the ration of synthesized metabolites is changing. Furthermore, deuterium induces physiological, morphological and cytological alterations in the cell. This leads to the formation in D_2O of large atypical cells. They are usually 2–3 times larger in size and have a thicker

cellular wall compared to the control cells grown on H₂O. The structure of DNA in deuterated cells in D₂O may alter; distribution of DNA in them was non-uniform. The data obtained confirm that adaptation to D₂O is a phenotypical phenomenon as the adapted cells return back to normal growth after some log-period after their replacement into H₂O. At the same time the effect of convertibility of growth on H₂O/D₂O does not exclude an opportunity that a certain genotype determines displaying of the same phenotypical attribute in D₂O.

Experiments with D₂O have shown (figure 1), that green-blue algae is capable to grow on 70% D₂O, methylotrophic bacteria – 75% D₂O, chemoheterotrophic bacteria – 82% D₂O, and photoorganoheterotrophic bacteria – 95 % D₂O.

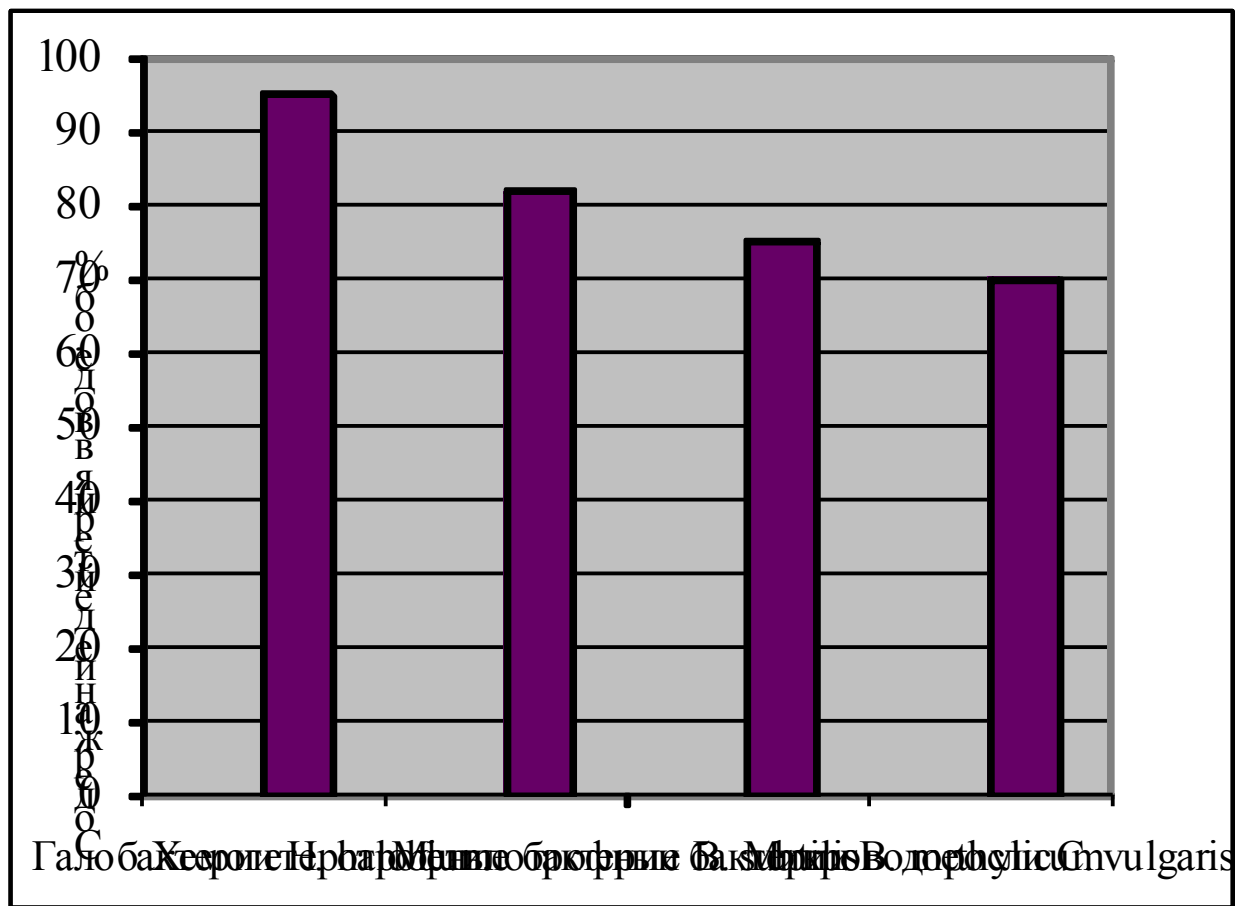


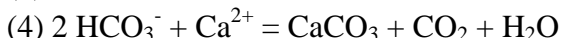
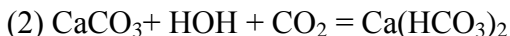
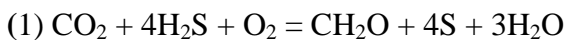
Figure. 1. Survival rate of cells of the studied microorganisms in water with various content of deuterium.

In the process of adaptation to D₂O the most important for macromolecular structure are dynamic short-lived hydrogen (deuterium) bonds formed between the neighbor atoms of H(D) and O, C, N, S-heteroatoms, playing an essential role in maintenance of spatial structure of macromolecules and intermolecular interactions. The substitution of H with D atom affects the stability and geometry of hydrogen bonds in apparently rather complex way and may, through the changes in the hydrogen bond zero-point vibrational energies, alter the conformational dynamics of hydrogen (deuterium)-bonded

structures of DNA and protein in D₂O. It may cause disturbances in the DNA-synthesis, leading to permanent changes in DNA structure and consequently in cell genotype. The multiplication which would occur in macromolecules of even a small difference between a proton and a deuteron bond 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 D₂O. And next, the changes in dissociation constants of DNA and protein ionizable groups when transferring the macromolecule from H₂O to D₂O may perturb the charge state of the DNA and protein molecules. Other important property is defined by the three-dimensional structure of D₂O molecule having the tendency to pull together hydrophobic groups of macromolecules to minimize their disruptive effect on the hydrogen (deuterium)-bonded network in D₂O. This leads to stabilization of the structure of protein and nucleic acid macromolecules in the presence of D₂O. At placing a cell in D₂O, not only H₂O is removed from a cell due to reaction of D₂O dissociation, but also there is occurred fast isotopic (H-D) exchange in hydroxyl (-OH), sulfohydryl (-SH) and amino groups (-NH₂) of all 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 substances with bonds such as C-D can be synthesized de novo.

Biological experiments with D₂O and structural-conformational studies enable to modeling conditions under which life has evolved. The most favorable are accepted alkaline mineral waters interacting with CaCO₃ and then sea waters. Once appeared in these waters the process of self-organization of primary organic forms in water solutions may be supported by thermal energy of magma, volcanic activity and solar radiation.

In connection with these data are important the following reactions:



The equation (1) shows how some chemosynthetic bacteria use energy from the oxidation of H₂S to S. The equation (2) is related to formation of Ca(HCO₃)₂ from H₂O, CO₂ and CaCO₃. In the presence of hydroxyl OH⁻ ions CO₂ transforms into HCO₃⁻ (equation (3)). Equation (4) is valid for the process of dolomite formation of stromatolites.

Furthermore, we have carried out the research of mineral, sea and mountain water from Bulgaria by IR-spectroscopy method of differential non-equilibrium energy spectrum (DNES) relative to the control – deionized water (fig. 2, curves 1-5, the table). In experiments were investigated samples of water from karst springs. Also IR-spectra of castus juice were investigated by DNES method (fig. 2, curve 1). The cactus was selected as a model system because the plant contains about 90% water. The closest to the IR-spectrum of castus juice was the IR-spectrum of the mineral water contacting with CaCO₃ (fig. 2, curve 2). IR-spectra of plant juice, mineral water and water of the kars springs have magnitudes of peaks in IR-spectra at -0,1112; -0,1187; -0,1262; -0,1287 and -0,1387 eV, accordingly. Similar peaks in the IR-spectrum between cactus juice, mountain and sea water were detected at -0,1362 eV. The IR-spectrum of the control sample of deionized water (fig. 2, curve 5) was substantially different from the IR-spectrum of sea mineral and mountain water. The values of average energy (ΔE_{H...O}) of hydrogen H...O-bonds between molecules H₂O

in the process of formation of $(H_2O)_n$ associates, measured by the DNES method were measured at $0,1067 \pm 0,0011$ eV.

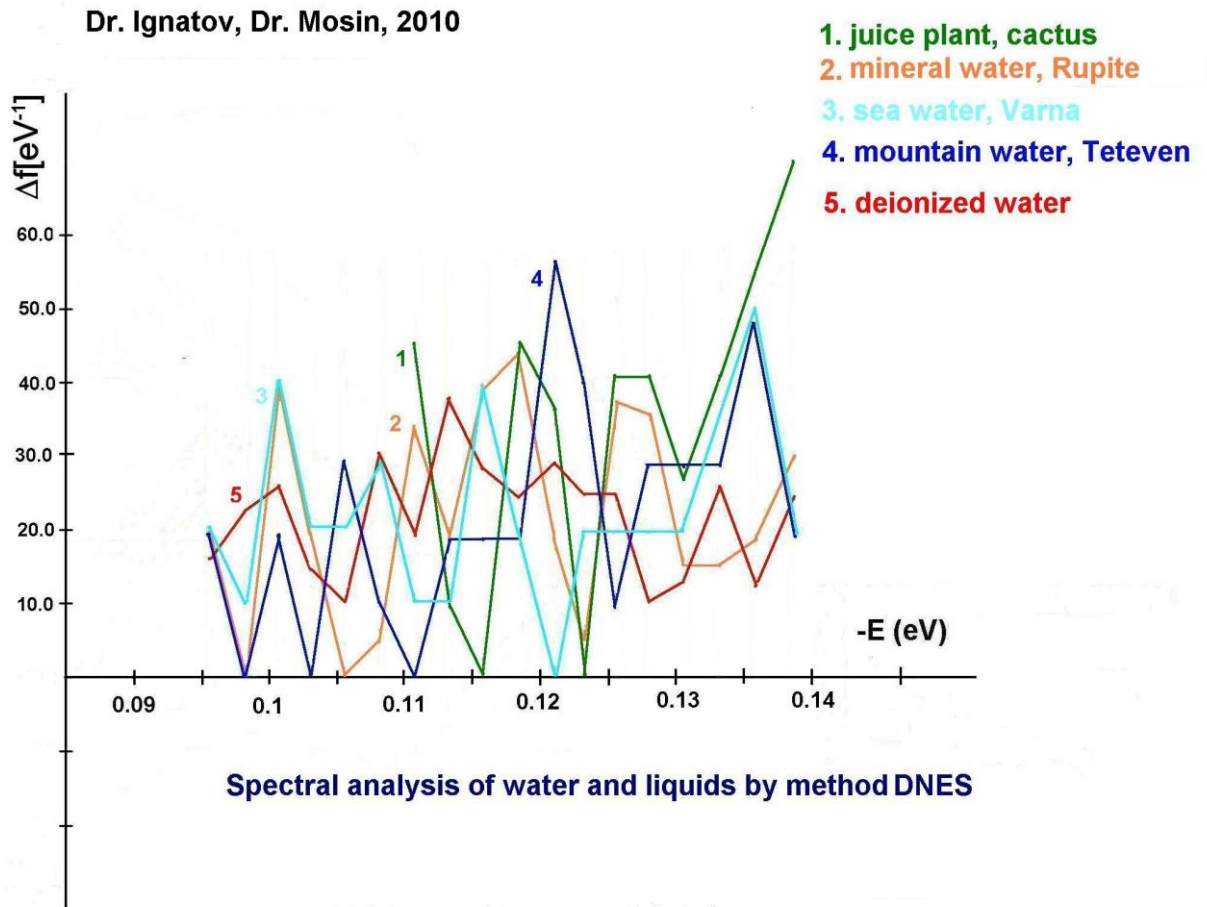


Figure. 2. DNES spectra of water of various origin: 1 – cactus juice; 2 – mineral water Rupite (Bulgaria); 3 – sea water (Varna, Bulgaria); 4 – mountain water (Teteven, Bulgaria); 5 – deionized water (control).

The table. Characteristics of IR-spectra of water of various origin obtained by DNES-method.

$-E_x$ (eV)	$-E$ (eV) Mineral water Rupite	$-E$ (eV) Sea water	μm	cm^{-1}
0,1112	0,1112		11,15	897
0,1187	0,1187		10,45	957

0,1262	0,1262		9,83	1017
0,1287	0,1287		9,64	1037
0.1362		0,1362	9,10	1099
0,1387	0,1387		8,95	1117

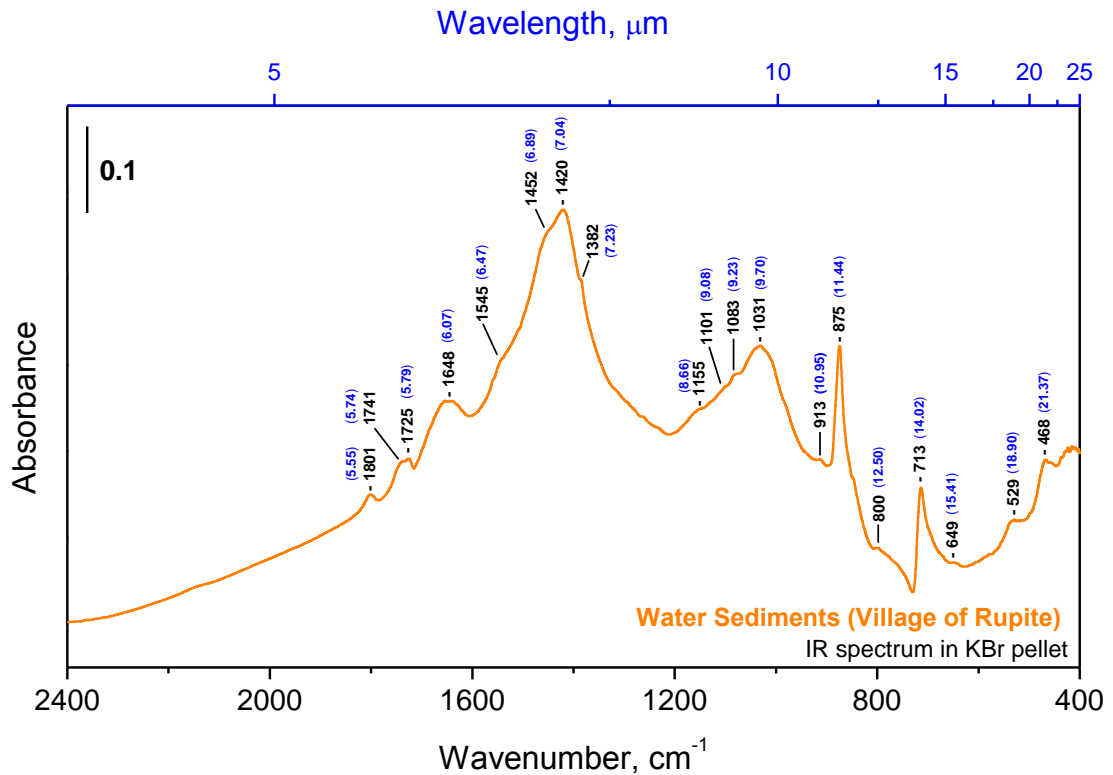


Figure.3. Results of infrared spectrum of Rupite, Bulgaria,

The spectrum of water in Rupite has been studied with infrared spectroscopy method with the device Thermo Nicolet Avatar 360 Fourier-transform IR. The study was carried out by Christina Chakarova, Institute of Physicochemistry, Bulgarian Academy of Sciences. At 9.7 μm or -0.1287 eV obtained value of local maximum. A value of local maximum at 9.64 μm or -0.1278 eV was obtained with the infrared spectroscopy method DNES. The statistical reliability of the DNES method is ± 0.0011 eV. The local maximum obtained with the DNES method at 9.83 μm (-0.1262 eV) and 8.95 μm (-0.1387 eV) are located on the spectral curve of the local maximum 9.7 μm (-0.1287 eV). The data obtained proved that hot mineral alkaline water is preferable for maintenance of life. These data also can predict a possible way of transition from synthesis of small organic molecules due to the energy of UV solar radiation and thermal activity to more complex organic molecules as protein and nucleic acids. The important factor in reaction of condensation of two molecules of amino acids is allocation of H_2O molecule when peptide chain is formed. As reaction of polycondensation of amino acids is accompanied by dehydration, the H_2O removal from reactional mixture speeds up the reaction rates. This testifies that formation of organic forms may occur

nearby active volcanoes, because at early periods of geological history volcanic activity occurred more actively than during subsequent geological times. However, dehydration accompanies not only amino acid polymerization, but also association of other blocks into larger organic molecules, and also polymerization of nucleotides into nucleic acids. Such association is connected with the reaction of condensation, at which from one block removes proton H^+ , and from another – hydroxyl group (OH^-) with formation of H_2O molecule.

The possibility of existence of condensation-dehydration reactions under conditions of primary hydrosphere was proven by Calvin in 1965 [11]. From most chemical substances hydrocyanic acid (HCN) and its derivatives – cyanoamid ($HNCN_2$) and dicyanoamid ($HN(CN)_2$) possess dehydration ability and the ability to catalyze the process of linkage of H_2O from primary hydrosphere [12]. The presence of HCN in primary hydrosphere was proven by Miller's early experiments. Chemical reactions with HCN and its derivatives are complex with chemical point of view; in the presence of HCN, $HNCN_2$ and $HN(CN)_2$ the condensation of separate blocks of amino acids accompanied by dehydration, can proceed at normal temperatures in strongly diluted H_2O -solutions. Furthermore, polycondensation of amino acids in the presence of HCN and its derivatives depends on acidity of water solutions in which they proceed [13]. In acid water solutions (pH 4-6) these reactions do not occur, whereas alkaline conditions (pH 8–9) promote their course. There has not been unequivocal opinion, whether primary water was alkaline, but it is probable, that such a value of pH possessed mineral waters adjoining with basalt, and these reactions could occur at contact of water with basalt rocks. Armen Mulikidjanian and Michael Galperin, were based on an analysis of the content of cellular elements, were concluded that in all probability life has not was originated in the ocean [15].

In synthesis of organic molecules other energy sources, e. g. geothermal sources could be used. Thus, in solutions of formaldehyde CH_2O with hydroxylamine NH_2OH , formaldehyde with hydrazine (N_2H_4) in water solutions with HCN, after heating of a reactionary mixture to $95^{\circ}C$ amino acids were detected [14]. In other experiments reaction products were polymerized into peptide chains that is the important stage towards inorganic synthesis of protein. In a reactionary mixture with solution HCN in water NH_3 were formed purines and pyrimidines [16]. In other experiments amino acid mixtures were subjected to influence of temperatures from $60^{\circ}C$ up to $170^{\circ}C$ with formation of short protein-like molecules resembling early evolutionary forms of proteins subsequently designated as thermal proteinoids [17]. They are consisted of 18 of 22 amino acids usually occurring in protein hydrolyzates. The synthesized proteinoids are similar to natural proteins on a number of other important properties, e. g. on linkage by nucleobases and ability to cause the reactions similar to those catalyzed by enzymes in living organisms as decarboxylation, amination, deamination, and oxidoreduction. Proteinoids are capable to catalytically decompose glucose [18] and to have an effect similar to the action of α -melanocyte-stimulating hormone [19]. Under certain conditions in hot mixture of proteinoids in water solutions are formed elementary membrane like proteinoid microspheres with diameter 5-10 μm [20]. On morphological features proteinoid microspheres remind a cellular membrane which may be as well double. In 2011 a team of Japanese scientists led by T. Sugawara created a membrane like proto cells from aqueous solution of organic molecules, DNA and synthetic enzymes under temperature close to water's boiling point $95^{\circ}C$ [20]. These laboratories experiments are an excellent confirmation of the possibility that life originated in hot water.

4. The conclusion

The data obtained testify that life maintenance depends on physical-chemical properties of water and external factors – temperatures, pH. Hot mineral alkaline water, which interacts with CaCO_3 is closest to these conditions. Next in line with regard to quality is sea and mountain water. In warm and hot mineral waters IR-peaks in DNES spectra were more expressed in comparison with the IR-peaks received in the same water with lower temperature. The spectral range of DNES was in the middle infrared range from 8 to 14 μm . It is thought that there is the Earth atmosphere's window of transparency for the electromagnetic radiation in the close and middle infrared range. In this interval energy is radiated from the Sun towards the Earth, and from the Earth towards surrounding space. If in the primordial hydrosphere was much more deuterium, this is a significant fact regarding thermal stability of deuterated macromolecules in the preservation of life under thermal conditions.

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