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Articles and Statements

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BLUP Values of Birth to Weaning Growth Traits of Awassi Lambs in Iraq

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Abstract

This study was carried out on the research station of sheep and goat (Abo Gharib, west of Baghdad, Iraq) to investigate the effects of some environmental factors on some growth traits in Awassi lambs as well as estimating heritability of these traits. Records of Awassi lambs included 1318 birth weights (BW), 821 weaning weights (WW) and 821 pre-weaning daily gains (DG) as recorded over three years (2007–2009). Records belonged to 120 sires. Restricted Maximum Likelihood (REML) method in a mixed model (paternal half sib) was utilized to estimate the heritability (h^2) for above mentioned traits. The general statistical model included fixed effects due to parity, sex, year and month of birth, type of birth and age at weaning as covariate. The Best Linear Unbiased Prediction (BLUP) values of sires for all studied traits were estimated. Results showed that the means of BW, WW and DG were 3.85, 26.64 and 0.181 kg respectively. Studied traits were significantly ($P < 0.01$) affected by all fixed effects except the effect of sire on WW and DG, which was significant ($P< 0.05$). Heritability estimates (h^2) of BW, WW and DG were 0.23, 0.12 and 0.19 respectively. The lowest and highest BLUP values for the mentioned traits were -0.325, 0.255 kg, -1.142, 1.284 kg, and -0.103, 0.053 kg respectively.

Keywords: Awassi lamb, birth weight, weaning weight, heritability.

Introduction

Sheep husbandry in Iraq has been a historically important component of rural development and still fulfills a sustainable role in the livelihood of farmers. The country has a tradition of the consumption of sheep products, especially lamb and mutton. The native sheep breeds in Iraq include the Karadi (Kurdi, Hamdani, Jaff and Dzaie) 20%, Awassi (Naami and Shefali) 58.2% and Arabi 21.8% (Al-Barzinji and Othman, 2013). The Awassi sheep breed has been introduced into many countries, and have been shown to have superior performance to some native breeds (Todorovski, 1988). This breed was widespread because of its good characteristics in regards to

meat price and quality (Kingwell et al., 1995), milk quality (Sunderman and Johns, 1994), validity of wool for the carpet industry (Lightfoot, 1988), and its ability to cope stress of high environmental temperature (AbiSaab and Sleiman, 1995). Unfortunately, the Awassi has taken its place among the genotypes of indigenous genetic resources requiring a protection project due to their declining numbers (Üstüner and Oğan, 2013). Today on sheep farming, a large part of the economic income is based on meat production. Consequently, it is observed that studies aimed to increase lambs productivity and growth performance in lambs, which are the main source of meat production, have intensified (Özcan et al., 2001).

In order to devise effective breeding plans for genetic improvement of Awassi sheep, information on the extent of genetic and environmental factors on performance traits is the pre-requisite. Therefore, this study was planned to generate information on the relative importance of genetic and environmental factors on the growth performance of Awassi sheep in addition to estimate the heritability and Best Linear Unbiased Prediction (BLUP) for some growth traits.

Material and methods

In this study, records of Awassi lambs bred at the research station of sheep and goats (Abu Gharib, west of Baghdad, Iraq) were utilized over three years (2007–2009). Data included 1318 birth weight (BW), 821 weaning weight (WW) and 821 pre-weaning daily gain (DG) records. The numbers of ewes and rams were 849 and 120 respectively.

Restricted Maximum Likelihood (REML) method in a mixed model (paternal half sib) was used to estimate the heritability (h^2) for mentioned traits. The general statistical model included fixed effects due to parity, sex, year of birth, month of birth, type of birth and age at weaning as covariate.

PROC mixed in SAS program (2010) was used to estimate Best Linear Unbiased Prediction (BLUP) values of sires for all studied traits.

SAS program Ver. 9.1 was used for data analysis. Two Mathematical models were assumed as shown below:

Model I for BW:

$$Y_{ijklmno} = \mu + Pi + Yj + Sk + Tl + Mm + F_n + e_{ijklmno}$$

where; Y_{ijklm} = Birth weight; μ = Population mean; Pi = Effect of parity (1–3); Yj = Year of birth, 2007, 2008, 2009; Sk = Sex, Male, female; Tl = Type of birth (Single, Twin); Mm = Month of birth (January, February, and March); $e_{ijklmno}$ = random error associated with each observation. It is assumed to be normally distributed with mean zero and variance σ^2.

Model II for WW and DG:

As weaning lambs in this station depend on weight rather than age, the WW and DG were adjusted for age at weaning.

$$Y_{ijklmno} = \mu + Pi + Yj + Sk + Tl + Mm + Fn + b(X_{ijklmn}) + e_{ijklmno}$$

The notation of the second model is similar to the first model except b = regression coefficient; X_{ijklmn} = Age of lamb at weaning.

Results and discussion

Least square means ± SE of BW, WW and DG of the Awassi lambs in relation to parity, month and year of lambing, type of birth and sex are presented in Table 1. The overall means of BW, WW and DG were 3.85±0.02 kg, 26.64±0.25 kg and 0.181±0.003 kg respectively.

Table 1: Lease square means ±SE (kg) for birth weight (BW) (kg), weaning weight (WW) and daily gain (DG) in Awassi lambs

Factor	No	BW	No	WW	DG
Parity					
1st	549	3.67±0.14 b	239	26.82±0.88 a	0.164±0.015 b
2nd	539	4.19±0.11 a	354	28.73±0.68 a	0.191±0.012 a
3rd	230	4.28±0.16 a	228	23.48±1.09 b	0.139±0.012 c
Year of birth					
2007	528	3.64±0.12 b	222	31.13±0.88 a	0.221±0.013 a
2008	574	3.78±0.12 b	383	24.06±0.64 b	0.141±0.013 b
2009	216	4.68±0.17 a	216	23.84±1.16 c	0.132±0.013 b
Month of birth					
January	326	4.25±0.04 a	233	29.18±0.36 a	0.202±0.005 a
February	804	4.03±0.02 b	483	26.20±0.24 b	0.179±0.004 b
March	188	3.83±0.05 c	105	23.65±0.52 c	0.151±0.007 c
Type of birth					
Single	676	4.33±0.03 a	416	28.21±0.29 a	0.189±0.004 a
Twin	642	3.74±0.03 b	405	24.47±0.30 b	0.166±0.004 b
Sex					
Male	653	4.15±0.03 a	398	27.77±0.31 a	0.189±0.004 a
Female	665	3.92±0.03 b	423	24.92±0.29 b	0.166±0.003 b
Reg. on age at weaning				0.174±0.019**	0.003±0.0001**
Overall mean	1318	3.85±0.02	821	26.64±0.25	0.181±0.003

Means of the same column with different letters differ significantly at $P < 0.01$.
** ($P < 0.01$)

Estimates of heritability (h^2) and BLUP values are presented in Table 2. The heritability of the BW, WW and DG was 0.23, 0.19 and 0.12 respectively.

The BLUP values of sires for the studied traits are shown in Table 2. The lowest BLUP values for BW, WW, and DG were -0.325, -1.142 and -0.103 kg respectively. The corresponding values of highest BLUP values were 0.225, 1.284, and 0.053 kg.

Table 2. Heritability estimates and BLUP values of birth weight, weaning weight, and pre-weaning daily gain (kg) in Awassi sheep

		Birth weight		Weaning weight		Daily gain
Heritability		0.23±0.04		0.12±0.04		0.19±0.04
Sequence	Sire No.	BLUP	Sire No.	BLUP	Sire No.	BLUP
1	12345	-0.32520	11996	-1.14220	11873	-0.10330
2	11920	-0.26250	11877	-0.90160	11877	-0.09137
3	11975	-0.24560	11958	-0.79060	11878	-0.06243
4	1239	-0.15690	11873	-0.76560	11958	-0.03495
5	11911	-0.15100	11941	-0.66780	11961	-0.02903
-	-	-	-	-		
-		-	-	-		
116	11916	0.17260	11948	0.57440	11914	0.02901
117	11925	0.19210	11908	0.63980	11931	0.03187
118	11978	0.19350	11914	0.77440	11989	0.03926
119	11964	0.21480	11913	0.77760	1748	0.04575
120	12241	0.25520	11989	1.28400	11913	0.05321

The mean of the BW in current study was lower than the range of the means (4.05 to 4.52 kg) reported by several researchers for Awassi lambs (Esenbuğa and Dayıoğlu, 2002; Hassen et al., 2004; Dikmen et al., 2007; Jawasreh and Khasawneh, 2007; Kridli et al., 2007; Tabbaa et al., 2008; Üstüner and Oğan, 2013). However, it is consistent with other estimates of 3.82, 3.70 and 3.67 kg reported by Al-Kass et al. (1986), Al-Wahab (2003) and Al-Khazrji et al. (2014) respectively.

The findings of the current research that sire had significant ($P < 0.01$) effect on BW is similar to those obtained by Al-Hilali (1982), and Alkass et al. (1991). The parity affected the BW significantly ($P < 0.01$). The mean of BW increased with advancing parity and this could be attributed to increasing of ewe's weight. According to Křížek et al. (1983), live weight of dams significantly affected live weight of lambs at birth. Hence, balanced feeding for dam could lead to the heavier lambs at birth (Obaido, 2010). Similar finding was reported by Ghoneim et al. (1982) and Al-Khazrji et al. (2014).

The effect of year of birth on the BW was significant ($P < 0.01$). This effect reflects the variation of the availability of rainfall and pastures among different years. The month of birth also influenced BW. Lambs born early in the lambing period (January and February) surpassed those born later (March). This could be attributed to variation in quantity and quality of dam's nutrition through the gestation period particularly in the last months. Similar results were reported by Khalaf et al. (2010), who found a similar significant ($P< 0.01$) trend where BW was 3.82, 3.46 and 3.24 kg for January, February and March respectively.

The type of birth and sex affected BW significantly ($P < 0.01$). Results showed that the birth weight was higher in males compared to females and in single-born lambs compared to twins. These results were in consistent with those reported by Dikmen et al. (2007). Singles and males generally had higher birth weights than twin births and females. These results confirmed the result obtained by Üstüner and Oğan (2013).

Results revealed that the mean of the WW (26.64kg) was lower than means of 31.29, 29.14 and 27.75 kg reported by Özcan et al. (2001), Üstüner and Oğan (2013), and Khalaf et al. (2010) respectively. On the other hand it is higher than the value of 21.54 to 24.30 kg reported by Al- Jalili et al. (2006), Al-Wahab (2003), Aksakal et al. (2009) and Al-Salman (2009).

WW differed significantly ($P < 0.01$) among parities where it was heaviest in the 2nd parity than those born in the 1st and 3rd parities.

This could be attributed to lamb growth rate, which is mainly affected by the dams' milk yield. High milking ewes' lambs grow faster as compared to the poor milkers (Obaido, 2010). This result was in agreement with results obtained by Al-Salman (2009) and Al-Khazrji et al. (2014), but disagreed with others (Jawasreh and Khasawneh, 2007; Üstüner and Oğan, 2013).

There was a significant ($P <0.01$) decreasing in the WW a cross years of birth. Year of birth within locations generally showed highly significant effects on BW, WW, and yearling weight. Fluctuations in some environmental factors prevailed; in particularly quality and quantity of the available feed stuff could be an explanation (AKF, 2006).

The month of birth also influences WW. Lambs born early in the lambing period (January and February) will gain weight better than those born late (March) due to the accessibility to the pastures in spring season. Lambs born late will not be able to use pasture in spring because of their young age besides they may have higher exposure to internal parasites which thrive in the high temperature. Thus, their weaning and yearling weights will be lower than those lambed early (Elwakil et al., 2009).

The differences in WW between the months of lambing were attributed to the yearly variation in the rain precipitation and its effect on the density, growth and availability of pastures, forage, and other feeds. Similarly, different climates have been reported to influence milk production of ewes. This indirectly affects the growth of lambs (Shaker et al., 2002). The results achieved in this study are in congruency with Al-Salman (2009), who found that WW of lams born in November and December had significantly ($P< 0.05$) heavier WW (25.98 and 25.74 kg) as compared with those born in January and February (22.63 and 20.85 kg).

In the current study, the type of birth and sex affected significantly ($P< 0.01$) the WW. The WW was higher in males compared to females and in single-born lambs compared to twins. Similar finding was obtained by Dikmen et al. (2007), Al-Salman (2009), and Üstüner and Oğan (2013).

The regression coefficient of WW on age at weaning was positive and significant ($P < 0.01$). Thus, it is an important to adjusting WW for this variation in age at weaning.

The present estimate of DG (0.181 kg) appeared to be comparable with the corresponding estimates of Awassi lambs (0.168 to 0.205 kg) reported by other researchers (Al-Salman, 2009; Üstüner and Oğan, 2013; Al-Khazrji et al., 2014).

The impact of all fixed effects on DG was significant ($P < 0.01$). The trend of changes in DG due to fixed effects is parallel to changes in WW. This could be attributed to the effect of the DG on WW.

Paternal half-sib heritability for BW, WW, and DG was given in Table 2. Their values were considered low to moderate. Heritability estimates of 0.10, 0.19, 0.18, and 0.16 for BW of Awassi lambs were reported by Kazzal (1973), Thrift et al. (1973), Aziz (1977), and Gursoy et al. (1995). The higher estimates of 0.29 and 0.41 for the same breed were obtained by Al-Hilali (1982), and Alkass et al. (1991). The heritability estimate of the BW in the present study indicated that sires differed in their genetic potential.

The heritability estimate of the WW (0.12) was much lower than the estimate of BW. This could be attributed to variation in maternal environment mainly milk supply rather than to differences in genetic merit of the lambs. This estimate was similar to those (0.10, 0.12 and 0.10) reported by Thrift et al. (1973), Dzakumah et al. (1978) and Alkass et al. (1991).

In current work, heritability of DG (0.19) was higher than 0.12 and 0.07 that reported by Al-Rawi et al. (1982) and Kamber (1987) for the same breed, while our estimate was lower than 0.51 reported by Alkass et al. (1991). Differences in heritability estimates among various studies for the same trait of the same breed could be due to differences in the records number used, the correction for different non-genetic factors, the model used and the methodology for estimating heritability of the trait (Abou-Bakr, 2009).

With lowest BLUP values (Table 2), this indicates that selection for BW would be effective in raising the BW of Awassi lambs, particularly the heritability of BW was moderate. The expected genetic gain per generation under mass selection would be the product of the selection differential and heritability. On the other hand the differences between highest and lowest BLUP values for WW and DG were not high enough to justify selection procedures for their improvement particularly the two traits have low heritability.

Results of the year of birth effects in the present study indicated that while BW generally tends to increase along with advancing years, the WW and DG tend to decrease. Despite the breeding plan implemented in this flock, the management as well as the effectiveness of the breeding plan has to be considered in order to improve the mutton production.

Conclusion

Results of the present study showed that the heritabilities of growth traits in Awassi lambs ranged from the low to moderate estimations. In other words, it refers that the most variation in the phenotypic traits belonged to environmental factors. However, the BLUP values point to considerable differences existed among the rams and that could be invested for improving growth traits by selection elite rams.

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Наилучший линейный несмещенный прогноз (BLUP) значений роста от рождения до вскармливания ягнят породы Авасси в Ираке

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Аннотация. Исследование проведено на научно-исследовательской ветеринарной станции (Або Гариб, запад Багдада, Ирак) для исследования влияния некоторых факторов окружающей среды на рост породы ягнят Авасси, а также оценки наследуемости этих признаков. Параметры включали 1318 значений веса при рождении (BW), 821 значений веса при отъеме от кормления (WW) и 821 значений ежедневной прибыли веса до отъема от кормления (DG), зарегистрированные в течение трех лет (2007–2009). Исследования

производились с 120 бычками породы Авасси. Для оценки наследуемости (h^2) для вышеназванных признаков был использован метод ограниченного максимального правдоподобия (REML) в смешанной модели (отцовская половина мужского потомства). Статистическая модель включала способность к деторождению, пол, год, месяц рождения и возраст при отъеме от кормления. Оценивался наилучший линейный объективный прогноз (BLUP) значений для всех изученных признаков. Результаты показали, что значения BW, WW и DG соответствуют 3.85, 26.64 и 0.181 кг соответственно. Изученный признаки были значительно ($P < 0,01$) подвержены влиянию всех эффектов, за исключением влияния на WW и DG, который был значительным ($P < 0.05$). Оценки наследуемости (h^2) BW, WW и DG составили 0.23, 0.12 и 0.19 соответственно. Самые низкие и самые высокие значения BLUP для указанных признаков составили -0.325, 0.255 кг, -1.142, 1.284 кг и -0.103, 0.053 кг соответственно.

Ключевые слова: ягнята Авасси, вес при рождении, вес при отъеме от вскармливания, наследственность.

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Multifactorial Research of Longevity Phenomenon in Mountainous and Field Areas of Bulgaria

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Abstract

The article outlines the data on longevity factors and mountain water in factorial research of phenomenon of longevity in mountainous and field areas of Bulgaria. It was established the dependence among various internal and external factors on a phenomenon of longevity – the residence area, health status, gender and heredity. Natural waters derived from various Bulgarian water springs as well as water with varying deuterium content and the human blood serum of cancer patients were investigated by IR, NES and DNES methods. As estimation factor was measured the values of the average energy of hydrogen bonds ($\Delta E_{H...O}$) among H_2O molecules, as well as local maxima in the IR, and DNES-spectra of various samples of water and human blood serum at $\Delta E_{H...O}$ = -0,1387 eV (the DNES-method) and λ = 8,95 μm (the IR-method). 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 with the reduced deuterium content until the deep removal of deuterium (60–100 ppm) has

beneficial effects on human health. For a group of people in critical condition of life and patients with malignant tumors the greatest values of local maxima in DNES-spectra are shifted to lower energies relative to the control group.

Keywords: deuterium, heavy water, deuterium depleted water, longevity, mountain water, IR, NES, DNES.

Introduction

The question of longevity has always been an exciting one for humanity. Aging is a biological process, which leads to reduction of the vital functions of the body, limiting its adaptive capacities, and development of age-related pathologies and ultimately increasing the likelihood of death, is a part of the normal ontogeny and is caused by the same processes that lead to increased functional activity of various body systems in earlier periods of life. It is possible that these processes along with other processes (growth and development of the organism, etc.) are programmed in the human genome and biological mechanism of regulation. The question to what extent aging is dependent on heredity is not sufficiently proven in modern science.

Like other biological processes, aging is accelerated under the influence of certain exogenous and endogenous factors and occurs in different individuals with different speed, which depends on genetic differences and environmental factors. The best chance for longevity gives the longevity of immediate direct genetic ancestors. That is why the direct descendants of centenarians generally have the best chances for longevity. O. Burger demonstrated that life expectancy has increased substantially from the 19^{th} to the 20^{th} century and that this cannot be advantageously associated with the human genome [1]. The main factors of longevity are water quality, food and improved advancement of medicine. For example, in Bulgaria the average life expectancy from 1935 to 1939 was 51,75 years, while from 2008 to 2010 it was 73,60 years. In Russia, the average life expectancy in 2012 has reached 69 years.

From the standpoint of genetics, the process of aging is associated with the disruption of the genetic program of the organism and gradual accumulation of errors during the process of DNA replication. Aging may be associated with the accumulation of somatic mutations in the genome and be influenced by free radicals (mainly oxygen and primary products of oxidative metabolism) and ionizing radiation on DNA molecules as well [2]. Such mutations can reduce the ability of cells to the normal growth and division and be a cause of a large number of various cell responses: inhibition of replication and transcription, impaired cell cycle division, transcriptional mutagenesis, cell aging that finally result in cell death. Cells taken from the elderly people show a reduction in transcription when transferring information from DNA to RNA.

From the standpoint of dynamics, aging is a non-linear biological process, which increases over time. Accordingly, the rate of aging increases with time. The accumulation of errors in the human genome increases exponentially with time and reaches a certain stationary maximum at the end of life. This is most possible that, for this reason, the probability of cancer occurrence increases with age. According to thermodynamics, the process of aging is the process of alignment of the entropy by the human body with that of the environment [3].

Water is the main substance of life. The human body is composed from 50 to 75 % of water. With aging, the percentage of water in the human body decreases. Hence, the factor of water quality is the essential factor for the research. Water is present in the composition of the physiological fluids in the body and plays an important role as an inner environment in which the vital biochemical processes involving enzymes and nutrients take place. Water is the main factor for metabolic processes and aging. Earlier studies conducted by us, have demonstrated the role of water, its structure, isotopic composition and physical-chemical (pH, temperature) in the growth and proliferation of prokaryotes and eukaryotes in water with different isotopic content [4, 5]. These factors and the structure of water are of great importance for biomedical studies. The peculiarities of chemical structure of H_2O molecule create the favorable conditions for formation of electrostatic intermolecular Van-der-Waals forces, dipole-dipole forces and donor-acceptor interaction with transfer of charges between H-atom and O-atoms in H_2O molecules, binding them into water associates (clusters) with the general formula $(H_2O)_n$ where n varies from 3 to 50 units [6]. Other important indicator of water quality is its isotopic composition. The natural water consists on 99,7 mol.% of $H_2{}^{16}O$, which molecules are formed by ^{1}H and ^{16}O atoms [7]. The remaining 0,3 mol.% is represented by isotope varieties (isotopomers) of water molecules,

wherein deuterium forms 6 configurations of isotopomers – $HD^{16}O$, $HD^{17}O$, $HD^{18}O$, $D_2{}^{16}O$, $D_2{}^{17}O$, $D_2{}^{18}O$, while 3 configuration are formed by isotopomers of oxygen – $H_2{}^{16}O$, $H_2{}^{17}O$, $H_2{}^{18}O$.

This report studies the influence of various internal and external factors on a phenomenon of longevity – residence area, health status, gender, heredity, isotopic composition of water with using non-equilibrium (NES) and differential non-equilibrium (DNES) spectrum of water. The research was carried out under the joint scientific project "*Nature, Ecology and Longevity*" conducted in Bulgaria. In frames of this project 217 people living in the municipalities of Teteven, Yablanitza and Ugarchin, Lovech district (Bulgaria), where is lived the most number of long living people and their siblings, were studied. They have the same heredity, but have lived under different conditions. In all three municipalities there is a mountainous and a field part. Mountain and tap water is used for drinking. Statistical analysis has been conducted for heredity, body weight, food, diseases, positive attitude towards life.

Material and methods

Objects of studying

The objects of the study were various prokaryotic and eukaryotic cells of microorganizms obtained from the State Research Institute of Genetics and Selection of Industrial Microorganisms (Moscow, Russia). Experiments were also carried out with the samples of natural mountain water from various Bulgarian springs and human blood serum.

Preparation of water samples with varying deuterium content

For preparation of water samples with varying deuterium content we used D_2O (99,9 atom%) received from the Russian Research Centre "Isotope" (St. Petersburg, Russian Federation). Inorganic salts were preliminary crystallized in D_2O and dried in vacuum before using. D_2O was distilled over $KMnO_4$ with the subsequent control of deuterium content in water by ^{1}H-NMR-spectroscopy on Brucker WM-250 device ("Brucker", Germany) (working frequency – 70 MHz, internal standard – Me_4Si). The melt water was obtained from Moscow tap water by the freeze-thaw method in a standard procedure: 1,5 l of Moscow tap water was placed in a glass jar with a lid and placed in the refrigerator freezer at -14 ^{0}C for 4–5 hours. Then, the first ice crystals were mechanically removed from the mixture, and the jar again was placed in the freezer additionally for 8–10 hours before ¾ of liquid freezes. Thereafter, the liquid brine is decanted and the remaining ice was thawed at room temperature and used for further experiments. The melt water was stored in a glass container in refrigerator. Other experiments were carried out with deuterium depleted water (DDW) with residual deuterium content of 60–100 ppm, purchased from Langway Water Inc. (Moscow).

Studying the Bulgarian centenarians

Interviews have been conducted with 415 Bulgarian centenarians and long lived people and their siblings. Their heredity, body weight, health status, tobacco consumption, physical activity, attitude towards life has been analyzed. With using DNES method was performed a spectral analysis of 15 mountain water springs located in municipalities Teteven and Kuklen (Bulgaria). The composition of water samples was studied in the laboratory of "Eurotest Control" (Bulgaria). Statistics methods were attributed to the National Statistical Institute of Bulgaria.

Studying the human blood serum

1 % (v/v) solution of human blood serum was studied with the methods of IR-spectroscopy, non-equilibrium (NES) and differential non-equilibrium (DNES) spectrum. The specimens were provided by Kalinka Naneva (Municipal Hospital, Bulgaria). Two groups of people between the ages of 50 to 70 years were tested. The first group (control group) consisted of people in good clinical health. The second group included people in critical health or suffering from malignant diseases.

DNES spectral analysis

The device for DNES was made from A. Antonov on an optical principle. In this study was used a hermetic camera for evaporation of water drops under stable temperature (22±24 ^{0}C)

conditions. The water drops are placed on a water-proof transparent pad, which consists of thin Mylar folio and a glass plate. The light is monochromatic with filter for yellow color with wavelength λ = 580±7 nm. The device measures the angle of evaporation of water drops from $72,3^0$ to 0^0. The spectrum of hydrogen bonds among H_2O molecules was measured in the range of 0,08–0,1387 eV or λ = 8,9–13,8 μm using a specially designed computer program. The main estimation criterion was the average energy ($\Delta E_{H...O}$) of hydrogen O...H-bonds between H_2O molecules in human blood serum.

IR-spectroscopy

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

Statistical processing of experimental data

Statistical processing of experimental data was performed using the statistical package STATISTISA 6 using the Student's *t*- criterion (at $p < 0,05$).

Results and discussions

Comparative analysis between longevity of centenarians and their siblings

In frames of the research 54 long living people from Bulgaria over 90 years of age have been studied together with their siblings. The average lifespan of Bulgarian centenarians is 89,1 years, and for their brothers and sisters the average lifespan is 87,8 years. The difference in life expectancy of the two groups of people is reliable and is at $p < 0,05$, *t*-Student's criteria at a confidence level of t = 2,36 years.

There are 21519 residents in Teteven and 142 of them were born before 1924. Figure 1 show the interrelation between the year of birth of long living people (age) and their number (Teteven municipality, Bulgaria).

Figure 1: Interrelation between the year of birth of long-living people (age) and their number in Teteven municipality, Bulgaria.

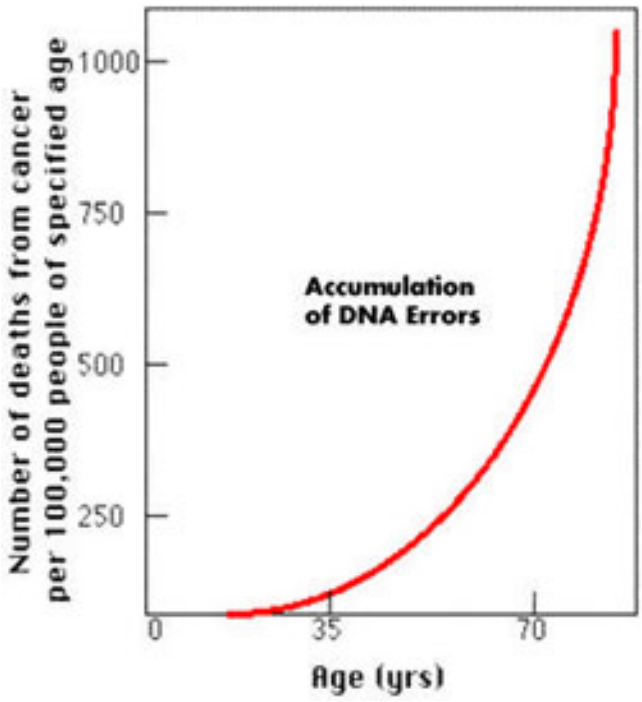

Figure 2: Interrelation between age and the number of cancer patients.

It was shown in Figure 1 that the rate of aging increases with time. In 1963 L. Orgel showed that the aging process is associated with the synthesis of abnormal proteins [8]. Figure 2 shows L. Orgel's results on the interrelation between age and number of cancer patients. The accumulation of errors in synthesis of abnormal proteins increases exponentially over time with age. Cells taken from elderly people show the reduced levels of transcription or transmission of information from DNA to RNA. Therefore, the probability of cancer increases with age. The interrelation between the number

of Bulgarian centenarians in the mountainous municipality of Teteven and their age is close to exponential.

Empirical evidence on life duration

Human experience shows that long-living people inhabit mainly high mountainous areas where flow the rivers feed by mountain springs. In Russia the most number of centenarians live in Russian North and Dagestan region (Russian Federation). One explanation for this is that water in those places contains less deuterium than ordinary drinking water [9].

In 1989 G. Berdishev studied the phenomenon of longevity of centenarians in Yakutia and Altai regions (Russian Federation) [10]. He linked the longevity of the Yakuts and the Altaians with the consumption of melt water from glaciers formed earlier in Yakutia's mountains than these of Greenland. According to the State's statistics most of the Russian centenarians live in Dagestan and Yakutia – 353 and 324 persons per 1 million inhabitants. This number for all Russia is only 8 people for 1 million. In Bulgaria the average number of centenarians makes up 47 per 1 million, while in Teteven Municipality – 139 centenarians per 1 million. In the Bulgarian municipalities the oldest inhabitant in field areas is 93 years old, and the oldest inhabitant in mountainous areas is 102 years old. There are distances of no more than 50 km between these places and the only difference is mountain water and air.

Here are submitted the data for Bulgaria:

- Varna district – centenarians 44 per 1 million, plain and sea regions;
- Pleven district – centenarians 78 per 1 million, plain regions;
- Teteven district – centenarians 279 per 1 million, hills and mountainous regions;
- Bulgaria – centenarians 47 per 1 million.

Analogous situation is observed in the Russian North. According to G. Berdishev, people inhabiting the Russian North – the Yakuts and the Altaians as well as the Buryats, drink predominantly the mountain water obtained after the melting of ice (melt-water). Altai and Buryat water sources are known as moderately warm, with temperatures of +8–10 ^{0}C, the water is generally ice-free in winter. This phenomenon is explained by the fact that the melt water contains a low percentage of deuterium compared with ordinary tap water that is believed to have a positive effect on the tissue cells and metabolism. The melted water in Russia is considered to be a good folk remedy for increasing physical activity of the human body, enhancing the vitality of the organism and has a beneficial effect on metabolism [11].

The mountain water in springtime is the result of the melting of ice and snow accumulated in the mountains. Natural ice (ice I_h, hexagonal lattice) is usually much cleaner than water, because the solubility of all substances (except NH_4F) in ice is extremely low. The growing ice crystal is always striving to create a perfect crystal lattice and therefore displaces impurities. The melt water obtained after the thawing of ice has a certain "ice-like" structure, because it preserves the hydrogen bonding between water molecules; as a result it is formed complex intermolecular associates (clusters) – the analogues of ice structures, consisting of a different number of H_2O molecules (Fig. 3). However, unlike the ice crystal, each associate has a very short time of existence as a result there occurs the constant processes of decay and formation of water associates having very complicated structure [13]. The specificity of intermolecular interactions characteristic for the structure of ice, is kept in melt water, as it is estimated that in the melting of ice crystal is destroyed only 15% of all hydrogen bonds in the associates. Therefore, the inherent to ice connection of each H_2O molecule with four neighboring H_2O molecules is largely disturbed, although there is observed the substantially "blurring" of oxygen lattice framework. Processes of decay and formation of clusters occur with equal probability that is probably why physical properties of melt water are changed over time, e.g. dielectric permittivity comes to its equilibrium state after 15–20 min, viscosity – in 3–6 days [14]. The heating of fresh melt water above t = +37 ^{0}C leads to a loss of the biological activity. The storage of melted water at +22 ^{0}C is also accompanied by a gradual decrease in its biological activity; within ~16–18 hours it is reduced by 50%. The main difference between the structure of ice and water is more diffuse arrangement of the atoms in the lattice and disturbance of long-range order. The thermal oscillations (fluctuations) lead to the bending and breaking down of hydrogen bonds. H_2O molecules being out of equilibrium positions begin to "fall down" into the adjacent structural voids and for a time held up there, as cavities correspond to the relative minimum of potential energy. This

leads to an increase in the coordination number, and the formation of lattice defects. The coordination number (the number of nearest neighbors) during the transition from ice to melt water varies from 4,4 at +1,5 ^{0}C to 4,9 at +80 ^{0}C.

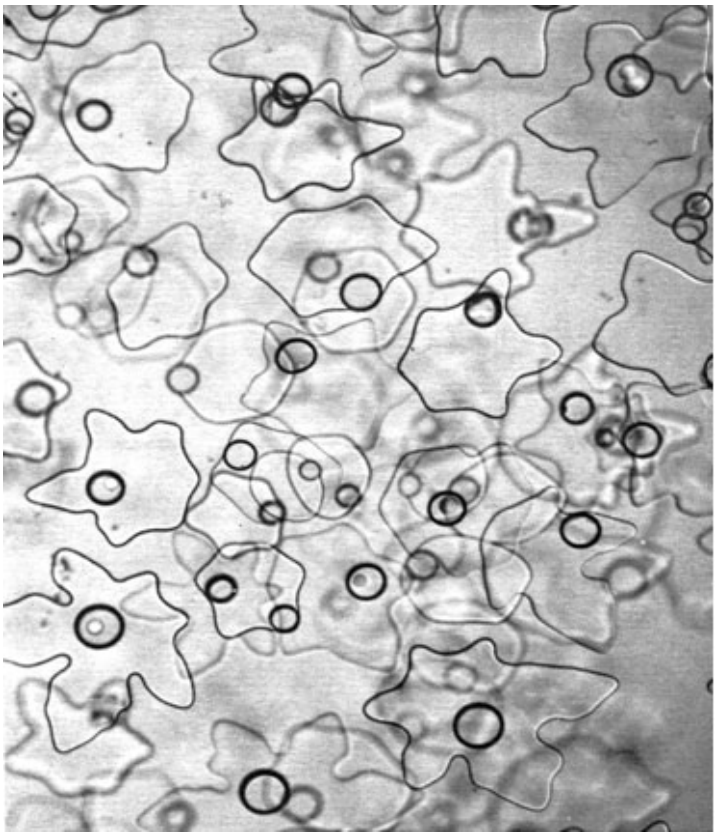

Figure 3: Structure of melt water containing “smearing” fragments of regular hexagonal ice structures according to computer simulations.

Preliminary analyses of water from various water sources show that the melt water obtained by the freeze-thaw method as well as mountain water contain less amount of deuterium as the result of natural isotope purification. The melt water also contains ions of Ca^{2+}, Mg^{2+}, Na^{+}, HCO_3^{-} and SO_4^{2+}. The content of K+ and N+ cations in the melt water is approximately 20–25 mg/l, Mg^{2+} – 5–10 mg/l, Ca^{2+} – 25–30 mg/l, the content of SO_4^{2-} – <90 g/l, HCO_3^{-} 50–100 mg/l, Cl^{-} – less than 70 mg/l, total rigidity ≤ 5 mEq/l, the total mineralization ≤ 0,3 g/l, pH – 6,5–7,0 at t = +25 ^{0}C (Table 1). The degree of natural purification of melt water from impurities makes up ~55–60%. The concentration of salts of rigidity – Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, heavy metals and organochlorine compounds, as well as heavy isotopes, including deuterium in melt water is less that of ordinary portable water. This fact is important because some authors consider the hardness of the water to be among the main factors in cardiovascular diseases [15]. However, the mild correlation was further proven that water hardness could not be a decisive factor for human longevity.

Table 1: Chemical composition of melt water obtained from tap water by the freeze-thaw method

Cations, mg/l	
$K^+ + Na^+$	20–25
Mg^{2+}	5–10
Ca^{2+}	25–30
Anions, mg/l	
SO_4^{2-}	<90
HCO_3^{-}	50–100
Cl^{-}	<50

Other physical characteristics	
Total rigidity, mEq/l	≤5
Total mineralization, g/l	≤0,2
pH at t =+25 ^{0}C	6,5–7,0
Deuterium content, ppm	~132,5

Analysis of water samples from various sources of Russia and Bulgaria show that the mountain water contains on average ~4–5 % less deuterium in form of HDO, than the river water and sea water. 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 SMOW and SLAP (Table 2). According to the international SMOW the standard (the oceanic water) the isotopic shifts for D and ^{18}O in seawater: D/H = (155,76±0,05)·10^{-6} (155,76 ppm) and ^{18}O/^{16}O = (2005,20±0,45)·10^{-6} (2005 ppm) [16]. For the SLAP standard (the Atlantic oceanic water) the isotopic shifts for D and ^{18}O in seawater: D/H = 89·10^{-6} (89 ppm) and for a pair of ^{18}O/^{16}O = 1894·10^{-6} (1894 ppm). In surface waters, the ratio D/H = ~(1,32–1,51)·10^{-4}, while in the coastal seawater – ~(1,55–1,56)·10^{-4}. Waters of other underground and surface water sources contain varried amounts of deuterium (isotopic shifts) – from δ = +5,0 D,%, SMOW (Mediterranean Sea) to to δ = -105 D,%, SMOW (Volga River). The natural waters of CIS countries are characterized by negative deviations from SMOW standard to (1,0–1,5)·10^{-5}, in some places up to (6,0–6,7)·10^{-5}, but there are also observed positive deviations at 2,0·10^{-5}. The content of the lightest isotopomer – $H_2{}^{16}O$ in water corresponding to the SMOW standard is 997,0325 g/kg (99,73 mol.%), and for the SLAP standard – 997,3179 g/kg (99,76 mol.%).

Table 2: 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
$^1H_2{}^{16}O$	18,01056470	997,032536356	997,317982662
$^1HD^{16}O$	19,01684144	0,328000097	0,187668379
$D_2{}^{16}O$	20,02311819	0,000026900	0,000008804
$^1H_2{}^{17}O$	19,01478127	0,411509070	0,388988825
$^1HD^{17}O$	20,02105801	0,000134998	0,000072993
$D_2{}^{17}O$	21,02733476	0,000000011	0,000000003
$^1H_2{}^{18}O$	20,01481037	2,227063738	2,104884332
$^1HD^{18}O$	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)

The thawed snow and glacial water in the mountains and some other regions of the Earth also contain less deuterium than ordinary drinking water. On average, 1 ton of river water contains 150–200 g of deuterium. The average ratio of H/D in nature makes up approximately 1:5700. According to the calculations, the human body throughout life receives about 80 tons of water containing in its composition 10–12 kg of deuterium and associated amount of heavy isotope ^{18}O. That is why it is so important to purify the drinking water from heavy isotopes of D and ^{18}O.

Clinical evidence on the benefits of deuterium depleted water (DDW) for health

When biological objects exposed to water with different deuterium content, their reaction varies depending on the isotopic composition of water and magnitude of isotope effects determined by the difference of constants of chemical reactions rates k_H/k_D in H_2O and D_2O. The maximum

kinetic isotopic effect observed at ordinary temperatures in chemical reactions leading to rupture of bonds involving hydrogen and deuterium lies in the range $k_H/k_D = 5–7$ for C–H versus C–D, N–D versus N–D, and O–H versus O–D-bonds [17].

Our previous studies have shown that heavy water 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 stronger that those ones formed from hydrogen [18]. In mixtures of D_2O with H_2O with high speed occurs dissociation reactions and isotopic (H–D) exchange resulting in formation of semi-heavy water (HDO): $D_2O + H_2O = HDO$. For this reason deuterium presents in smaller content in aqueous solutions in form of HDO, while in the higher content – in form of D_2O. The chemical structure of D_2O molecule is analogous to that one for H_2O, with small differences in the length of the covalent H–O-bonds and the angles between them. D_2O boils at +101,44 ^{0}C, freezes at +3,82 ^{0}C, has density of 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.

The chemical reactions and biochemical processes in the presence of 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 (Table 3). 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. 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 [19]. 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 3: Changes in the physical properties of water with the isotopic substitution

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

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 [20]. It may cause disturbances in the DNA-synthesis, leading to permanent changes on DNA structure and consequently on cell genotype [21].

Our experiments demonstrated that the effects of deuterium on the cell possess a complex multifactor character connected to changes of physiological parameters – magnitude of the *lag*-period, time of cellular generation, outputs of biomass, a ratio of amino acids, protein, carbohydrates and fatty acids synthesized in D_2O, and with an evolutionary level of organization of investigated object as well. The cell evidently implements the special adaptive mechanisms promoting functional reorganization of work of the vital systems in the presence of D_2O.

D_2O can cause the metabolic disorders, kidney's malfunction, the violation of hormonal regulation and caused the immunosuppression [22], notwithstanding the strong radioprotective effect of D_2O [23]. Also deuterium induces physiological, morphological and cytological alterations on the cell with forming cells more 2–3 times larger in size in D_2O. At high concentrations of D_2O are suppressed enzymatic reactions, cell growth, mitosis and synthesis of nucleic acids [24]. 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 affecting simultaneously the functional condition of several numbers of cellular systems: metabolism, ways of assimilation of carbon substrates, biosynthetic processes, and transport function, structure and functions of macromolecules. Evidently cells 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 [25].

We have obtained results on growth and adaptation to D_2O of various cells of prokaryotic and eukaryotic organisms. Our studies have shown that animal cells are able to withstand up to 25–30% D_2O, plants – up to 50–60% D_2O, and protozoa cells are able to live on ~90% D_2O. Further increase in the concentration of D_2O for these groups of organisms leads to cellular death. On the contrary, DDW with the decreased deuterium content until the deep removal of deuterium (60–100 ppm) has beneficial effects on organism. Experiments on animals and plants demonstrated that after the consumption of DDW with the reduced on ~25–30% deuterium pigs, rats and mice produced a larger number of offspring, upkeep of poultry with 6-day old to puberty on DDW leads to accelerated development of the genital organs (size and weight), and strengthen the process of spermatogenesis, wheat ripens earlier and gives higher yields [26]. In addition, DDW delays the appearance of the first metastasis nodules on the spot inoculation of cervical cancer and exerts immunomodulatory and radioprotective effect [27].

Radioprotective effects of DDW were studied in reports [28] at irradiation of mice's cells by γ-radiation at semi-mortal dose LD_{50}. Survival level of animals treated with DDW 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 [29].

The consumption of DDW by cancer patients during or after radiation therapy treatments allows restore the composition of blood and relieve nausea [30]. According to G. Shomlai, the results of clinical trials conducted in 1998–2010 in Hungary showed that the survival rate for patients drinking DDW in combination with traditional therapies or after are significantly higher than for patients who only used the chemotherapy or radiation therapy [31].

The biological experiments with deuterium DDW with residual deuterium content of 60 ppm carried out in Moscow Research Oncological Institute after P.A. Herzen and N.N. Blokhin and Institute of Biomedical Problems, was confirmed the inhibitory effects of DDW on the process of growth of various tumors, e.g. division of the breast adenocarcinoma MCF-7 tumor cells placed in DDW started with a delay of ~5–10 hours [32]. In 60% of mice with the immunosuppressed immunity and transplanted human breast tumor MDA and MCF-7 consumption of DDW caused tumor regression. A group of mice with transplanted human prostate tumor PC-3 consumed DDW 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. In this regard special attention deserves two indicators: the delay of metastasis and loss of animal's weight during experiments. Stimulating action of DDW 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 DDW at the end of the experiment was 2 times less. It was also reported that DDW may delay the progression of prostate cancer [33] and inhibit the human lung carcinoma cell growth by apoptosis [34].

The preliminary experimental results on motility of human sperm, performed by V.I. Lobyshev and A.A. Kirkina [35], indicates that in DDW (4 ppm) the motility is on 40% higher during 5 hours of the registration. However, the effect depends on the initial properties of a sperm sample. These data indicated that deuterium content variation in water including deep deuterium depletion produce various nonlinear isotopic effect on key processes in a cell as 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 to inhibit fatty synthesis, chain elongation and desaturation. These anabolic reactions utilize acetyl-CoA, as well as hydrogen of water for new fatty acid pools [36]. 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 offer remarkable opportunities with altered morphology and flux handling properties.

The positive influence of drinking DDW on the 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 [37]. These data evidence the significance of DDW 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 DDW 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 [38]. On beneficial effect on health of experimental mice evidenced the increased resistance and weight increase compared with the control group [39]. It was also indicated that DDW increases the rate of metabolic reactions. It was observed the geroprotective (anti-aging), anti-mutagenic and radioprotective effects of DDW with reduced on 5% deuterium content on the development cycle of fruit fly *Drosophila melanogaster*.

The total effects of DDW depend on the following parameters – the total body mass, total body water, the amount of daily consumption of DDW and the degree of its isotope purification. The main impact of DDW on the organism is explained by gradual reduction of the deuterium content in the physiological fluids of the body by reactions of isotopic (H–D) exchange. These results indicate that the regular drinking of DDW helps improve the function of some vital systems [40]. With regular consumption of DDW there occurs the cleaning of organism from HDO due to the reaction of isotopic (H–D) exchange in physiological fluids, and it was recorded the change of the isotopic composition of urine and Ca^{2+} content as well. Daily consumption of DDW allows naturally reduce the content of HDO in the human body due to isotopic (H–D) exchange. It is believed that this process is accompanied by an increase in the functional activity of cells, cell tissues and organs. Thus regular consumption of DDW provides a natural way to reduce the content of HDO in the human body to lower values. It has beneficial effects on metabolism, invigorates the body, and also promotes the rapid recovery after strenuous physical exercise. This testifies the usage of DDW for residents of large cities.

Clinical trials of DDW (Langway Water Inc., Moscow) with a residual content of deuterium 60–100 ppm, showed 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, it was shown that 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, promotes weight correction, protects cells from radiation. 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.

The effectiveness of the influence of DDW depends on the following parameters – the total body mass, total body water, the amount of daily consumption of DDW and the isotopic content of deuterium. The results on the gradual increasing of deuterium content in the human body at regular consumption of DDW (Langway Water Inc., Moscow) with varied residual deuterium content of 60–100 ppm are shown in Table 4. This table shows that the content of deuterium in the human body decreases while drinking 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 drinking of DDW provides a natural way to reduce the content of HDO in the human body to a value of ~117 ppm.

Table 4: Gradual decreasing of deuterium content in the human body over time, with regular consumption of DDW (Langway Water Inc., Moscow)*

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 DDEW – 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).

Clinical evidence with human blood serum testing

A convenient method for studying of liquids is non-equilibrium differential spectrum. It was established experimentally that at the process of evaporation of water drops, the wetting angle θ decreases discreetly to 0, and the diameter of water drop basis is only slightly altered, that is a new physical effect. Based on this effect, by means of measurement of the wetting angle within equal intervals of time is determined the function of distribution of H_2O molecules according to the value of f(θ). The distribution function is denoted as the energy spectrum of the water state (ESWS). The theoretical research established the dependence between the surface tension of water and the energy of hydrogen bonds among individual H_2O-molecules.

For calculation of the function f(E) represented the ESWS, the experimental dependence between the wetting angle (θ) and the energy of hydrogen bonds between H_2O molecules (E) is established:

$$f(E) = \frac{14{,}33\, f(\theta)}{[1-(1+bE)^2]^2}, \qquad (1)$$

where $b = 14{,}33\ eV^{-1}$

The relation between the wetting angle (θ) and the energy (E) of the hydrogen bonds between H_2O molecules is calculated by the formula:

$$\theta = arcos\ (-1 - 14{,}33E), \qquad (2)$$

The energy spectrum of water is characterized by a non-equilibrium process of evaporation of water droplets therefore the term non-equilibrium spectrum (NES) of water is used.

The difference Δf(E) = f (samples of water) – f (control sample of water) - is called the "differential non-equilibrium energy spectrum of water" (DNES).

Thus, the DNES spectrum is an indicator of structural changes in water, because the energy of hydrogen bonds in water samples differ due to the different number of hydrogen bonds in water samples, which may result from the fact that different waters have different structures and composition and various intermolecular interactions - various associative elements etc. The redistribution of H_2O molecules in water samples according to the energy is a statistical process of dynamics.

We have conducted studies of 1 % (v/v) solution of human blood serum of two groups of people between 50 and 70 years of age by NES and DNES spectral analysis. The first group consisted of people in excellent health. The second group consisted of people in a critical state and patients with malignant tumors. The average energy of hydrogen bonds ($\Delta E_{H...O}$) between H_2O molecules in the blood serum was investigated as the main biophysical parameter. The result was registered as a difference between the NES-spectrum of 1 % solution of blood serum and NES-

spectrum of deionized water control sample – DNES-spectrum, measured as the difference Δf(E) = f (samples of water) – f (control sample of water). The DNES-spectrum obtained from the first group has a local maximum energy ($\Delta E_{H...O}$) at –9,1±1,1 meV and from the second group –1,6±1,1 meV. The results between the two groups have a statistical difference in *t*-Student's criterion at $p < 0,05$. For the control group of healthy people the value of the largest local maximum in the DNES-spectrum was detected at E = –0,1387 eV, or at λ = 8,95 μm. For the group of people in a critical state and the patients with malignant tumors, the analogous values of the largest local maximums of the DNES-spectrum shifted to lower energies compared with the control group of people. The norm has statistically reliable result for samples of human blood serum for the control group of people having cancer as the local maximum of function of distribution of H_2O molecules according to energy f(E) (eV^{-1}) in samples, which equals ~24,1 eV^{-1}.

In 1995 A. Antonov performed DNES-experiments with impact on tumor mice cells in water [41]. There was a decrease of the spectrum compared with the control sample of cells from a healthy mouse. The decrease was also observed in the spectrum of human blood serum of terminally ill people relative to that of healthy people (the control group). With increasing of age of long-living blood relatives, the function of distribution of H_2O molecules according to energies at –0,1387 eV decreases. In this group of tested people the result was obtained by DNES-method at *E* = –5,5±1,1 meV; the difference in age was of 20–25 years in relation to the control group. It should be noted that many of Bulgarian centenarians inhabit the Rhodope Mountains areas. Among to the DNES-spectrum of mountain waters similar to the DNES-spectrum of blood serum of healthy people at λ = 8,95 μm, was the DNES-spectrum of water in the Rhodopes. The mountain waters from Teteven, Boyana and other Bulgarian provinces have similar physical-chemical parameters.

The study the physiologic fluids (urine, blood, serum) by IR- and DNES-spectroscopy can also provide data on metabolic processes in the human body and longevity, because the IR- and DNES-spectra reflect the metabolic processes. It was demonstrated by the analysis of human blood serum by IR- and DNES-spectroscopy. The magnitude of the largest local maximum in IR-, DNES-spectra of human blood serum from healthy people of control group was observed at –E = 0,1387 eV (the DNES-method) and at λ = 8,95 μm (the IR-method). For a group of people in critical health condition and patients with malignant tumors the greatest values of local maxima in the IR-spectrum are shifted to lower energies relative to the control group. In IR-spectrum of human blood serum are detected 8 main local maxima at λ = 8,55; 8,58; 8,70; 8,77; 8,85; 9,10; 9,35 and 9,76 μm. The resulting peak at λ = 8,95 μm in the IR-spectrum of human blood serum approaching the peak at λ = 8,85 μm monitored by Russian researchers. In the control group of healthy people the average value of the energy distribution function f(E) measured by the DNES-method at λ = 8,95 μm compiles to 75,3 eV, and in a group of people in critical condition – 24,1 eV. The level of reliability of the results is $< 0,05$ according to the Student's *t*-criterion.

Comparatively, we studied the water samples of various Bulgarian water springs by the DNES-method. Table 5 shows the composition of the seven mountain springs in Teteven (Bulgaria) and local maximums in DNES-spectra of water samples. The local maximums were detected at E = –0,11 eV and E = –0,1387 eV. The value at E = -0,11 eV is characteristic for the presence of Ca^{2+} in water. The value at E = -0,1387 eV is characteristic for inhibiting the growth of cancer cells. Experiments conducted by A. Antonov with cancer cells of mice demonstrated a reduction of this local maximum to a negative value in water solutions containing Ca^{2+} (0,05 g/l). Analysis by the DNES-method of aqueous solutions of natural mineral sorbents – shungite (carbonaceous mineral from Zazhoginskoe deposit in Karelia, Russia) and zeolite (microporous crystalline aluminosilicate mineral from Most village, Bulgaria) showed the presence of a local maximum at –0,1387 eV for shungite and –0,11 eV for zeolite [42]. These results sugest the restructuring of energy values among H_2O molecules with a statistically reliable increase of local maximums in DNES-spectra. It should be noted that owing to the unique porous structures both the natural minerals shungite and zeolite are ideal natural water adsorbents effectively removing from water organo-chlorine compounds, phenols, dioxins, heavy metals, radionuclides, and color, and gives the water a good organoleptic qualities, additionally saturating it with micro-and macro-elements. It is worth to note that in Bulgaria the main mineral deposits of Bulgarian zeolites are located in the Rhodope Mountains, whereat has lived the greatest number of Bulgarian centenarians. It is believed that water in these areas is cleared in a natural way by zeolite. Therefore, a new parameter is entered

into Table 5 – a local maximum of energy at E = (-0,1362±0,1387 eV). This value is determined by the NES-spectrum as function of distribution of individual H_2O molecules according to energy f(E). The function of distribution of H_2O molecules according to energy f(E) (eV^{-1}) for tap water in Teteven is 11,8±0,6 eV^{-1}.

Table 5: The composition of mountain water springs in Teteven (Bulgaria) and local maximums in DNES-spectra of water

Sources	**Ca^{2+}**	**Na^{+}**	**Mg^{2+}**	**Fe^{2+}**	**SO_4^{2-}**	**pH**	**Local maximum* at -0,1362–0,1387)**
	mg/dm³ norm (<150)	mg/dm³ norm (<200)	mg/dm³ norm (<80)	mg/dm³ norm (<200)	mg/dm³ norm (<250)	norm (6,5–9,5)	eV^{-1} norm (>24,1)
1. Klindiovo	89,9±9,0	4,1±0,4	6,9±0,7	40,2±4,0	17,7±1,8	8,0±0,1	47,1±2,4
2. Gorna cheshma	103,6±10,1	4,2±0,4	15,5±1,6	9,6±0,96	89,9±9,0	7,8±0,2	20,0±1,0
3. Dolna cheshma	94,4±0,94	2,5±0,3	1,10±1,2	9,0±0,9	15,9±1,6	7,9±0,1	31,6±1,6
4. Sonda	113,6±11,4	7,3±0,7	15,9±1,6	5,00±0,5	57,2±5,7	7,3±0,1	48,8±2,4
5. Vila Cherven	–	–	–	–	13,3±1.3	7,5±0,1	44,4±2,2
6. Gechovoto	66,0±6,0	1,4±0,15	2,1±0,2	11,4±1,1	15,9±1,6	7,9±0,1	44,4±2,2
7. Ignatov izvor	40,4±3,1	0,6±0,1	2,46±0,2	13,0±1,4	17,9±1,8	6,8±0,1	31,6±1,6

Notes:
*Function of distribution of H_2O molecules in water samples according to energy f(E).

Heredity, stress, diet, smoking, body mass as additional longevity factors

The research showed that tobacco smoking increases the number of free radicals in the body [43]. The accumulation of free radicals leads to distortion of DNA replication. Evidently free radical-induced damage of DNA molecule plays an essential role in the process of aging. These data show that the average difference between the length of life of centenarians and their brothers is 10 of the 54 studied centenarians only 3 were long-time smokers (Table 6).

Table 6: Distribution of long living people and their sublings by gender

Number of centenarians	**Health status**	**Body mass**	**Smoking**	**Gender**	**Heredity**	**Positive attitude towards life**
54	In good health 48	Normal 54	Abstainers 51	Females 37	Parents and grandparents over 90 y.a. 18	54
0	With diseases 6	Above normal 0	Smokers 3	Males 17	No heredity 36	0

Table 7: Data for centenarians depending on their way of life

Number of centenarians	**Gender 20th and 21st century**	**Parents and grandparents over 20th and 21st century**
54	Females 37 Middle age 0	Females 15 Middle age 94,5
0	Male 17 Middle age 0	Male 13 Middle age 95,4

Table 7 shows an interesting trend, which, however, requires additional data for statistical analysis. In 2013 and 2014 the number of females was 69% and males – 21%. The number of parents and grandparents of long living people was 54% for females and 46% for males. The only two different factors were stress and probably smoking.

It is known that during the process of aging T-cell generation from the thymus is much reduced [44]. The decline rate of most T-cell and B-cell lymphocytes, which are crucial for the immune system, is faster in males than in the females. Furthermore, males showed a quicker decline in the two cytokines, IL-6 and IL-10 in relation to age. Two types of immune system cells, which annihilate external attackers, CD4 T-cells and natural killer (NK) cells are increased in number with age. The increase rate is higher in females than in males.

It should be noted that the process of aging can be limited if food caloricity of diet is being restricted on 40–55 %. In studies with 54 Bulgarian centenarians, all of them have had normal body mass throughout their lives; 48 of them were in excellent health condition, while 6 have various diseases. It is doubtful that these people would have reached longevity without being healthy. All of these studied people have had great physical activity. They live in friendly ecological environment in which the combination of mountain water, physical activity, diet and less stress are optimal for longevity. Further test has been created for the state of muscles, joints and tendons with prognostics for a longer life.

Conclusion

The experimental data shows that the direct relationship of man and nature – clean air, natural food from eco-farms and physical activity explains the difference between the larger number of centenarians who live in the mountain regions of Bulgaria and Russia and their high average number. Natural water with increased content of deuterium seems to be one of the most important factors for longevity. In Bulgaria, most centenarians live in the Rhodope Mountains, while in Russia – in Dagestan and Yakutia. Other longevity factors are living area, health status, body mass, gender and heredity. Studying the human blood serum by IR, NES and DNES-methods show that by measuring the average energy of hydrogen bonds among H_2O molecules and the distribution function of H_2O molecules according to energies it is possible to draw a vital state status of a person and the associated life expectancy. The IR-spectrum of the human blood serum of healthy group of people with a local maximum at $\lambda = 8{,}95$ μm is most similar to the IR-spectrum of the mountain water. The similar spectral characteristics possess mountain water from Teteven, Bojana and other Bulgarian sources. On the character of the IR-spectrum exerts an influence also the presence of deuterium. Thus, the phenomenon of longevity is a complex multi-factorial phenomenon involving both genetic (internal) and phenotypic (external) characteristics of the organism in its adaptation to the environment. Although additional data for parents and grandparents of long-living people are needed, total statistical analysis for all these summary factors will be essential for further scrutinized conclusions.

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Многофакторное исследование феномена долголетия в горных и равнинных областях Болгарии

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Аннотация. В работе представлены данные о факторах долголетия и горной воде как главном факторе долголетия в горных и равнинных районах Болгарии. Авторами установлена зависимость между различными внутренними и внешними факторами на феномен долголетия болгарских долгожителей, в том числе качеством потребляемой воды, изотопным составом воды, места жительства, состоянии здоровья, пола и наследственности. Природные воды из различных болгарских родников, а также вода с пониженным содержанием дейтерия и образцы сыворотки крови больных раком были исследованы методами ИК-спектроскопии, неравновесного энергетического (НЭС) и дифференциального неравновесного энергетического (ДНЭС) спектрального анализа. В качестве основного оценочного фактора использовали значения средней энергии водородных связей ($\Delta E_{H...O}$) между молекулами H_2O, а также локальные максимумы в ИК и ДНЭС-спектрах различных образцов воды и сыворотки крови человека при $\Delta E_{H...O}$ = -0,1387 эВ (ДНЭС-метод) и λ = 8,95

мкм (ИК-метод). Повышенное содержание дейтерия в воде приводит к физиологическим, морфологическим и цитологическим изменениям в клетках, а также оказывает негативное влияние на клеточный метаболизм, а вода с пониженным содержанием дейтерия, вплоть до глубокого удаления дейтерия (60–100 ppm) благотворно влияет на здоровье. Показано, что для группы людей в критическом состоянии жизни и больных со злокачественными опухолями наибольшие значения локальных максимумов в ДНЭС-спектрах смещаются в сторону меньших энергий по отношению к контрольной группе.

Ключевые слова: дейтерий, тяжелая вода, бездейтериевая вода, долголетие, горная вода, ИК, НЭС, ДНЭС.

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Studying Physiological, Morphological, and Cytological Alterations in Prokaryotic and Eukaryotic Cells in Heavy Water

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Abstract

In this paper the biological influence of deuterium on cells of various taxonomic groups of prokaryotic and eucaryotic microorganisms realizing methylotrophic, chemoheterotrophic, photo-organotrophic, and photosynthetic ways of assimilation of carbon substrates (methylotrophic bacteria *Brevibacterium methylicum*, chemoheterotrophic bacteria *Bacillus subtilis*, photo-organotrophic halobacteria *Halobacterium halobium*, and green algae *Chlorella vulgaris*) was investigated at the growth on media with heavy water (^2H_2O). For all investigated microorganisms are submitted data on growth and adaptation on growth media containing as sources of deuterated substrates ^2H_2O, [^{2}H]methanol and hydrolysates of deutero-biomass of methylotrophic bacteria *B. methylicum*, obtained after the multistage adaptation to ^2H_2O. The qualitative and quantitative composition of intra- and endocellular amino acids, proteins, carbohydrates and fatty acids in conditions of adaptation to ^2H_2O is investigated. It is shown, that the effects observed at adaptation to ^2H_2O, possess a complex multifactorial character and connected to physiological, morphological

and cytological alterations – the magnitude of the lag-period, time of cellular generation, output of biomass, a parity ratio of synthesized amino acids, proteins, carbohydrates and lipids, and also with an evolutionary level of the organization of the investigated object and the pathways of assimilation of carbon substrates.

Keywords: deuterium, heavy water, adaptation, isotopic effects, bacteria, micro algae.

Introduction

The most interesting biological phenomenon is the ability of some microorganisms to grow on heavy water (^2H_2O) media in which all hydrogen atoms are replaced with deuterium [1, 2]. ^2H_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 [3].

The average ratio of $^1H/^2H$ in nature makes up approximately 1:5700 [4]. In natural waters, the deuterium is distributed irregularly: from 0,02–0,03 mol.% for river water and sea water, to 0,015 mol.% for water of Antarctic ice – the most purified from deuterium natural water containing in 1,5 times less deuterium than that of seawater. According to the international SMOW standard isotopic shifts for ^2H and ^{18}O in sea water: $^2H/^1H = (155,76\pm0,05)\cdot10^{-6}$ (155,76 ppm) and $^{18}O/^{16}O = (2005,20\pm0,45)\cdot10^{-6}$ (2005 ppm). For SLAP standard isotopic shifts for ^2H and ^{18}O in seawater make up $^2H/^1H = 89\cdot10^{-6}$ (89 ppm) and for a pair of $^{18}O/^{16}O = 1894\cdot10^{-6}$ (1894 ppm). In surface waters, the ratio $^2H/^1H = \sim(1,32–1,51)\cdot10^{-4}$, while in the coastal seawater – $\sim(1,55–1,56)\cdot10^{-4}$. 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 however there are also observed the positive deviations at $2,0\cdot10^{-5}$.

The chemical structure of ^2H_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 ^2H_2O exceeds on 10% that one for H_2O. The difference in nuclear masses stipulates the isotopic effects, which may be sufficiently essential for the $^1H/^2H$ pair [5]. As a result, physical-chemical properties of ^2H_2O differ from H_2O: ^2H_2O boils at +101,44 ^{0}C, freezes at +3,82 ^{0}C, has maximal density at +11,2 ^{0}C (1,106 g/cm^3) [6]. In mixtures of ^2H_2O with H_2O the isotopic exchange occurs with high speed with the formation of semi-heavy water ($^1H^2HO$): $^2H_2O + H_2O = {}^1H^2HO$. For this reason deuterium presents in smaller content in aqueous solutions in form of $^1H^2HO$, while in the higher content – in form of ^2H_2O. The chemical reactions in ^2H_2O are somehow slower compared to H_2O. ^2H_2O is less ionized, the dissociation constant of ^2H_2O is smaller, and the solubility of the organic and inorganic substances in ^2H_2O is smaller compared to these ones in H_2O [7]. Due to isotopic effects the hydrogen bonds with the participation of deuterium are slightly stronger than those ones formed of hydrogen.

For a long time it was considered that heavy water was incompatible with life. Experiments with the growing of cells of different organisms in ^2H_2O show the toxic influence of deuterium. The high concentrations of ^2H_2O lead to the slowing down the cellular metabolism, mitotic inhibition of the prophase and in some cases – somatic mutations [8]. This is observed even while using natural water with an increased content of ^2H_2O or $^1H^2HO$ [9]. Bacteria can endure up to 90 % (v/v) ^2H_2O, plant cells can develop normally in up to 75 % (v/v) ^2H_2O, while animal cells – up to not more than 30 % (v/v) ^2H_2O [10]. The further increase in the concentration of ^2H_2O for these groups of organisms leads to the cellular death [11], although isolated cell's cultures suspended in pure ^2H_2O exert a strong radioprotective effect in ^2H_2O-solutions towards γ–radiation [12, 13]. On the contrary, deuterium depleted water with decreased deuterium content has benefitial effects on organism and stimulates the cellular metabolism [14].

With the development of new microbiological approaches, there appears an opportunity to use adapted to deuterium cells for preparation of deuterated natural compounds. The traditional method for production of deuterium labelled compounds consists in the growth on media containing maximal concentrations of ^2H_2O and deuterated substrates as [^{2}H]methanol, [^{2}H]glucose etc. [15, 16]. During growth of cells on ^2H_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 isolated from deuterated biomass obtained on growth media with high ^2H_2O content and deuterated substrates with using a combination of physico-chemical methods of separation – hydrolysis, precipitation, extraction with organic solvents and chromatographic purification by column chromatography on different adsorbents. These deuterated molecules evidently undergo structural adaptational modifications necessary for the normal functioning in ^2H_2O.

The adaptation to ^2H_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 [17]. 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 [18], IR spectroscopy [19] and mass spectrometry [20]. 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 on the adaptation to deuterium of various prokaryotic and eukaryotic organisms. 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.

Material and methods

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 photochrome transmembrane protein bacteriorhodopsin.
4. *Chlorella vulgaris B-8765*, photosynthesizing single-cell blue-green alga.

Chemicals

For preparation of growth media was used ^2H_2O (99,9 atom.%), ^{2}HCl (95,5 atom.%) and [^{2}H]methanol (97,5 atom% ^{2}H), purchased from the "Isotope" Russian Research Centre (St. Petersburg, Russian Federation). Inorganic salts and D- and L-glucose ("Reanal", Hungary) were preliminary crystallized in ^2H_2O and dried in vacuum before using. ^2H_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). According to ^{1}H-NMR, the level of isotopic purity of growth media usually was by ~8–10 atom% lower than the isotope purity of the initial ^2H_2O.

Adaptation technique

The initial microorganisms were modified by adaptation to deuterium by plating individual colonies onto 2 % (w/v) agarose growth media with stepwise increasing gradient of ^2H_2O concentration and subsequent selection of individual cell colonies stable to the action of ^2H_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 ^2H_2O (from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_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 ^{2}HCl (in ^2H_2O) and autoclaved for 30–40 min at 0,8 atm. The suspension was neutralized with 0,2 N KOH (in ^2H_2O) to pH = 7,0 and used as a source of growth substrates while adaptation and growing the chemoheterotrophic bacterium *B. sublilis* and the photo-organotrophic halobacterium *H. halobium*.

Growth media

The following growth media were used (concentratioin of components are given in g/l):

1. Minimal salt medium M9 for growth of the facultative methanol assimilating methylotrophic bacterium *B. methylicum B-5662*, supplemented with 2 % (v/v) [^{2}H]methanol and increasing gradient of ^2H_2O concentration from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_2O: KH_2PO_4 – 3; Na_2HPO_4 – 6; NaCl – 0,5; NH_4Cl – 1,0; L-leucine – 0,01.

2. Hydrolysated medium HM1 for growth of the aerobic Gram-positive chemoheterotrophic bacterium *B. subtilis B-3157*, based on ^2H_2O (89–90 atom% ^{2}H) and 2 % (w/v) hydrolysate of deuterated biomass of *B. methylicum B-5662* as a source of ^{2}H-labeled growth substrates: L-glucose – 120; hydrolysate of deuterated biomass of *B. methylicum* – 20, NH_4NO_3 – 20; $MgSO_4{\cdot}7H_2O$ – 10; $CaCO_3$ – 20; adenine – 0,01; uracil – 0,01. As a control was used protonated medium containing 2 % (w/v) yeast protein–vitamin concentrate (PVC).

3. Hydrolysated medium HM2 for the growth of the extreme aerobic halobacterium *Halobacterium halobium ET-1001* (based on 99,9 atom% ^2H_2O): NaCl - 250; $MgSO_4{\cdot}7H_2O$ - 20; KCl - 2; $CaCl_2{\cdot}\ 6H_2O$ – 0,065; sodium citrate – 0,5; hydrolyzate of deuterated biomass of *B. methylicum B-5662* – 20; biotin – $1{\cdot}10^{-4}$; folic acid – $1{,}5{\cdot}10^{-4}$, vitamin B_{12} – $2{\cdot}10^{-5}$.

4. Tamiya medium for the growth of the photosynthetic green microalgae *C. vulgaris B-8765* (based on 99,9 atom% ^2H_2O): 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; $ZnSO_4{\cdot}7H_2O$ – $4{\cdot}10^{-5}$; $CuSO_4{\cdot}5H_2O$ – $5{\cdot}10^{-5}$, $CoCl_2{\cdot}6H_2O$ – $5{\cdot}10^{-6}$).

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 λ = 620 nm. After 6–7 days the cells were harvested and separated by centrifugation (10000 g, 20 min) on T-24 centrifuge ("Heracules", Germany). The biomass was washed with ^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 culture liquids (CL) of appropriate strain-producers. Inosine was isolated from the 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 [21].

Protein hydrolysis

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

Hydrolysis of intracellular policarbohydrates

Dry biomass (50 mg) was placed into a 250 ml round bottomed flask, supplemented with 50 ml distilled ^2H_2O and 1,6 ml of 25 % (v/v) H_2SO_4 (in ^2H_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 ^2H_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.

Amino acid analysis

The amino acids of the hydrolyzed biomass were analyzed on a Biotronic LC-5001 (230×3,2) column ("Eppendorf–Nethleler–Hinz", Germany) with a UR-30 sulfonated styrene resin ("Beckman–Spinco", USA) as a stationary phase; the temperature – 20±25 ^{0}C; 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 λ = 570 and λ = 440 nm (for proline).

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 ("Water Associates", USA) using Ultrasorb CN column (250×10 mm) as a stationary phase; the temperature – 20±25 ^{0}C; the mobile phase – acetonitrile–water (75:25, % (w/w); the granule diameter – 10 μm; the input rate – 0,6 ml/min.

Analysis of fatty acids

Fatty acids were analyzed on a Beckman Gold System (USA) chromatograph (250×4.6 mm), equiped with Model 126 UV-Detector (USA), 20±25 ^{0}C. Stationary phase – Ultrasphere ODS 5 μm; mobile phase – linear gradient of 5 mM KH_2PO_4–acetonitrile; elution rate – 0,5 ml/min, detection at λ = 210 nm.

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. 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 ^2H_2O-media relative to the control obtained on H_2O.

Scanning electron 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 – ×150,000, resolution – 3,0 nm, accelerating voltage – 1–30 kV; sample size – 60–130 mm.

Results

Adaptation to deuterium the methylotrophic bacterium *B. methylicum*

Numerous studies with various biological objects in ^2H_2O proved that when biological objects are 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 ^2H_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–^{2}H, N–^{2}H versus N–^{2}H, and O–^{2}H versus O–^{2}H-bonds [22]. Isotopic effects

have an impact not only on the physical and chemical properties of deuterated macromolecules in which H atoms are substituted with ^2H atoms, but also on the biological behaviour of biological objects in ^2H_2O. Experiments with ^2H_2O (Table 1) have shown, that green-blue algae is capable to grow on 70 % (v/v) ^2H_2O, methylotrophic bacteria – 75 % (v/v) ^2H_2O, chemoheterotrophic bacteria – 82 % (v/v) ^2H_2O, and photo-organotrophic halobacteria – 95 % (v/v) ^2H_2O.

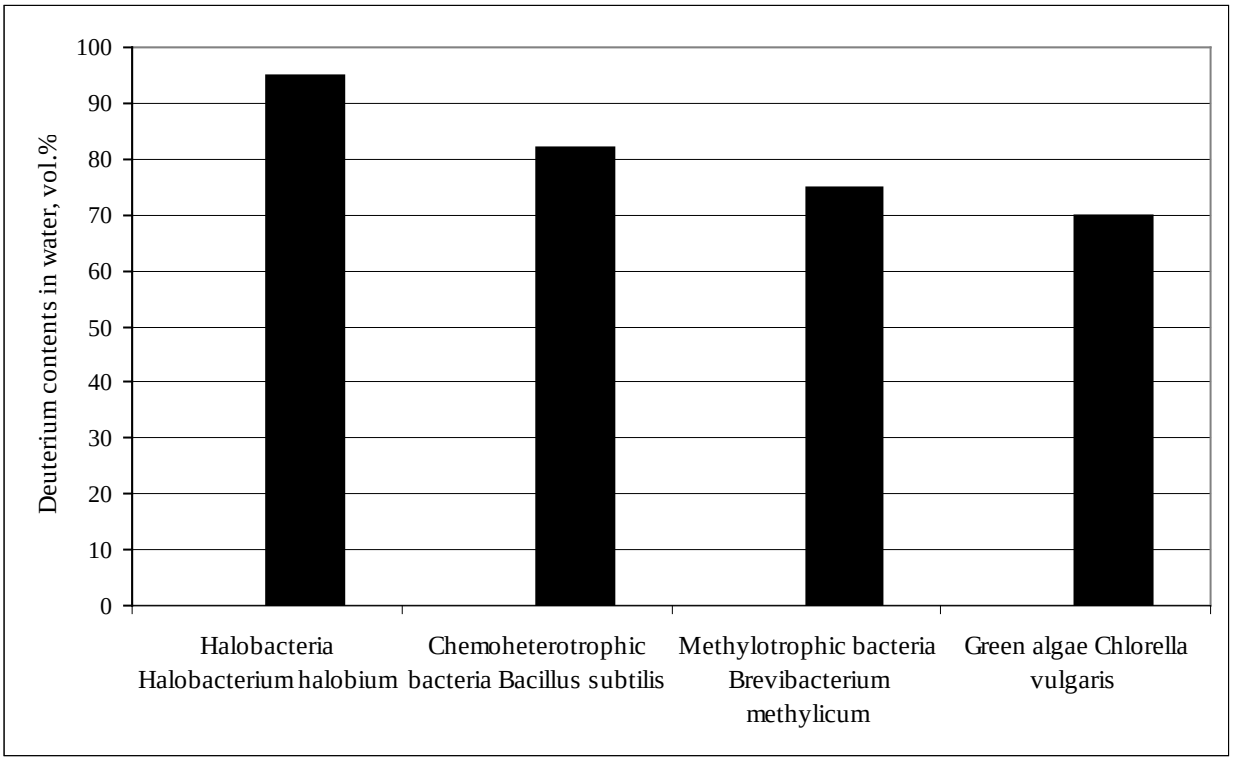


Figure 1: Cell survival of various microorganisms in water with different deuterium content (%, v/v)

In the course of the experiment were obtained adapted to the maximum concentration of ^2H_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 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 [^2H]methanol and ^2H_2O and its further use as a source of deuterated growth substrates for the growing other strains-producers in ^2H_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 [23]. 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.% ^2H) for biomedical use [24], 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.% ^2H) on growth media with high ^2H_2O-content.

We used stepwise increasing gradient concentration of ^2H_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 ^2H_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. For this, deuterium enrichment technique was applied *via* plating cell colonies on 2 % (w/v) agarose M9 media supplemented with 2 % (v/v) [U-^{2}H]MeOH with an increase in the ^2H_2O content from 0; 24,5; 49,0; 73,5 up to 98 % (v/v) ^2H_2O, combined with subsequent selection of cell colonies which were resistant to deuterium. The degree of cell survive on maximum deuterated medium was approx. 40 %. The data on the yield of biomass of initial and adapted *B. methylicum*, magnitude of lag-period and generation time on protonated and maximum deuterated M9 medium are shown in Figure 2. The yield of biomass for adapted methylotroph (*c*) was decreased approx. on 13 % in comparison with control conditions (*a*) at an increase in the time of generation up to 2,8 h and the lag-period up to 40 h (Figure 1). As is shown from these data, as compared with the adapted strain, the growth characteristics of initial strain on maximally deuterated medium were inhibited by deuterium.

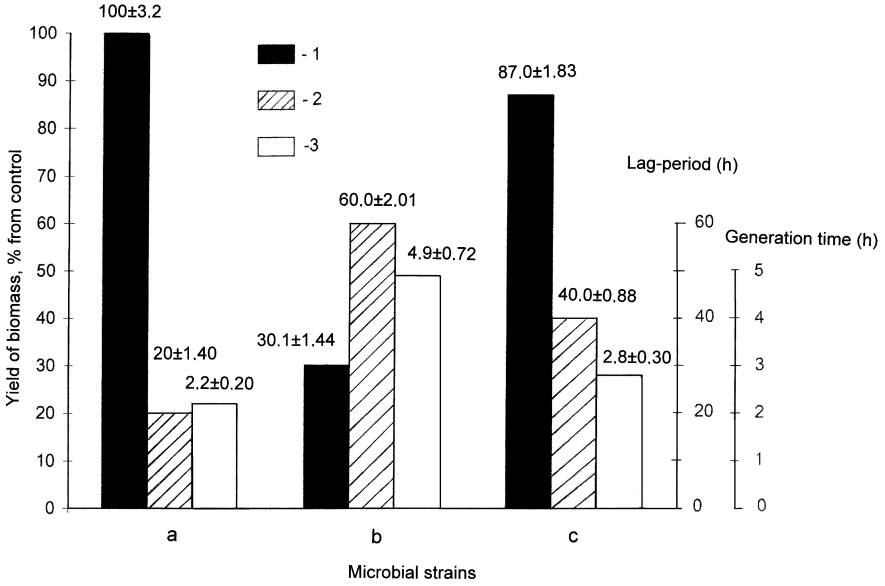


Figure 2: Yield of microbial biomass of *B. methylicum*, magnitude of lag-period and generation time in various experimental conditions: initial strain on protonated M9 medium (control) with water and methanol (*a*); initial strain on maximally deuterated M9 medium (b); adapted to deuterium strain on maximally deuterated M9 medium (c): 1 – yield of biomass, % from the control; 2 – duration of lag-period, h; 3 – generation time, h.

Experimental conditions are given in Table 1 (expts. 1–10) relative to the control (expt. 1) on protonated medium M9 and to the adapted bacterium (expt. 10'). Various compositions of [U-^{2}H]MeOH and ^2H_2O were added to growth media M9 as hydrogen/deuterium atoms could be assimilated both from MeOH and H_2O. The maximum deuterium content was under conditions (10) and (10') in which we used 98 % (v/v) ^2H_2O and 2 % (v/v) [U-^{2}H]MeOH. The even numbers of experiment (Table 1, expts. 2, 4, 6, 8, 10) were chosen to investigate whether the replacement of MeOH by its deuterated analogue affected growth characteristics in presence of ^2H_2O. That caused small alterations in growth characteristics (Table 1, expts. 2, 4, 6, 8, 10) relative to experiments, where we used protonated methanol (Table 1, expts. 3, 5, 7, 9). The gradual increment in the concentration of ^2H_2O into growth medium caused the proportional increase in lag-period and yields of microbial biomass in all isotopic experiments. Thus, in the control (Table 1, expt. 1), the duration of lag-period did not exceed 20,2 h, the yield of microbial biomass (wet weight) and production of phenylalanine were 200,2 and 0,95 gram per 1 liter of growth medium. The adding gradually increasing concentrations of ^2H_2O into growth media caused the proportional increasing of *lag*-period and yield of microbial biomass in all isotopic experiments. The results suggested, that below 49 % (v/v) ^2H_2O (Table 1, expts. 2–4) there was a small inhibition of bacterial growth compared with the control

(Table 1, expt. 1). However, above 49 % (v/v) ^2H_2O (Table 1, expts. 5–8), growth was markedly reduced, while at the upper content of ^2H_2O (Table 1, expts. 9–10) growth got 3,3-fold reduced. With increasing content of ^2H_2O in growth media there was a simultaneous increase both of lag-period and generation time. Thus, on maximally deuterated growth medium (Table 1, expt. 10) with 98 % (v/v) ^2H_2O and 2 % (v/v) [U-^{2}H]MeOH, lag-period was 3 fold higher with an increased generation time to 2,2 fold as compared to protonated growth medium with protonated water and methanol which serve as control (Table 1, expt. 1). While on comparing adapted bacterium on maximally deuterated growth medium (Table 1, expt. 10') containing 98 % (v/v) ^2H_2O and 2 % (v/v) [U-^{2}H]MeOH with non adapted bacterium at similar concentration showed 2,10 and 2,89 fold increase in terms of phenylalanine production and biomass yield due to deuterium enrichment technique, while, the lag phase as well as generation time also got reduced to 1,5 fold and 1,75 fold in case of adapted bacterium.

The adapted bacterium of *B. methylicum* eventually came back to normal growth at placing over in protonated growth medium after some lag-period that proves phenotypical nature of a phenomenon of adaptation that was observed for others adapted by us strains of methylotrophic bacteria and representatives of other taxonomic groups of microorganisms [25]. Literature reports clearly reveal that the transfer of deuterated cells to protonated medium M9 eventually after some lag period results in normal growth that could be due to the phenomenon of adaptation wherein phenotypic variation was observed by the strain of methylotrophic bacteria [26]. The improved growth characteristics of adapted methylotroph essentially simplify the scheme of obtaining the deutero-biomass which optimum conditions are M9 growth medium with 98 % ^2H_2O and 2 % [^{2}H]methanol with incubation period 3–4 days at temperature +35 ^{0}C.

Table 1: Effect of variation in isotopic content (0–98 % ^2H_2O, v/v) present in growth medium M9 on bacterial growth of *B. methylicum* and phenylalanine production

Bacterial strains	Exp. number	Media components, % (v/v)				Lag-period (h)	Yield in terms of wet biomass (g/l)	Generation time (h)	Phenylalanine production (g/l)
		H_2O	^2H_2O	MeOH	[U-^{2}H] MeOH				
Non adapted	1 (control)	98,0	0	2	0	20,2±1,40	200,2±3,20	2,2±0,20	0,95±0,12
Non adapted	2	98,0	0	0	2	20,3±1,44	184,6±2,78	2,4±0,23	0,92±0,10
Non adapted	3	73,5	24,5	2	0	20,5±0,91	181,2±1,89	2,4±0,25	0,90±0,10
Non adapted	4	73,5	24,5	0	2	34,6±0,89	171,8±1,81	2,6±0,23	0,90±0,08
Non adapted	5	49,0	49,0	2	0	40,1±0,90	140,2±1,96	3,0±0,32	0,86±0,10
Non adapted	6	49,0	49,0	0	2	44,2±1,38	121,0±1,83	3,2±0,36	0,81±0,09
Non adapted	7	24,5	73,5	2	0	45,4±1,41	112,8±1,19	3,5±0,27	0,69±0,08
Non adapted	8	24,5	73,5	0	2	49,3±0,91	94,4±1,74	3,8±0,25	0,67±0,08
Non adapted	9	98,0	0	2	0	58,5±1,94	65,8±1,13	4,4±0,70	0,37±0,06
Non adapted	10	98,0	0	0	2	60,1±2,01	60,2±1,44	4,9±0,72	0,39±0,05
Adapted	10'	98,0	0	0	2	40,2±0,88	174,0±1,83	2.8±0,30	0,82±0,08

Notes:

* The date in expts. 1–10 described the growth characteristics for non-adapted bacteria in growth media, containing 2 % (v/v) MeOH/[U-^{2}H]MeOH and specified amounts (%, v/v) ^2H_2O.

** The date in expt. 10' described the growth characteristics for bacteria adapted to maximum content of deuterium in growth medium.

***As the control used exprt. 1 where used ordinary protonated water and methanol

Adaptation, which conditions are shown in experiment 10' (Table 1) was observed by investigation of growth dynamics (expts. 1*a*, 1*b*, 1*c*) and accumulation of L-phenylalanine into

growth media (expts. 2*a*, 2*b*, 2*c*) by initial (*a*) and adapted to deuterium (*c*) strain *B. methylicum* in maximum deuterated growth medium M9 (Figure 3, the control (*b*) is obtained on protonated growth medium M9). In the present study, the production of phenylalanine (Figure 2, expts. 1b, 2b, 3b) was studied and was found to show a close linear extrapolation with respect to the time up to exponential growth dynamics (Figure 3, expts. 1a, 2a, 3a). The level of phenylalanine production for non-adapted bacterium on maximally deuterated medium M9 was 0,39 g/liter after 80 hours of growth (Figure 2, expt. 2b). The level of phenylalanine production by adapted bacterium under those growth conditions was 0,82 g/liter (Figure 3, expt. 3b). Unlike to the adapted strain the growth of initial strain and production of phenylalanine in maximum deuterated growth medium were inhibited. The important feature of adapted to ^2H_2O strain *B. methylicum* was that it has kept its ability to synthesize and exogenously produce L-phenylalanine into growth medium. Thus, the use of the adapted bacterium enabled to improve the level of phenylalanine production on maximally deuterated medium by 2,1 times with the reduction in the lag phase up to 20 h. This is an essential achievement for this strain of methylotrophic bacteria, because up till today there have not been any reports about production of phenylalanine by leucine auxotrophic methylotrophs with the NAD^+ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation. This makes this isolated strain unique for production of deuterated phenylalanine and other metabolically related amino acids.

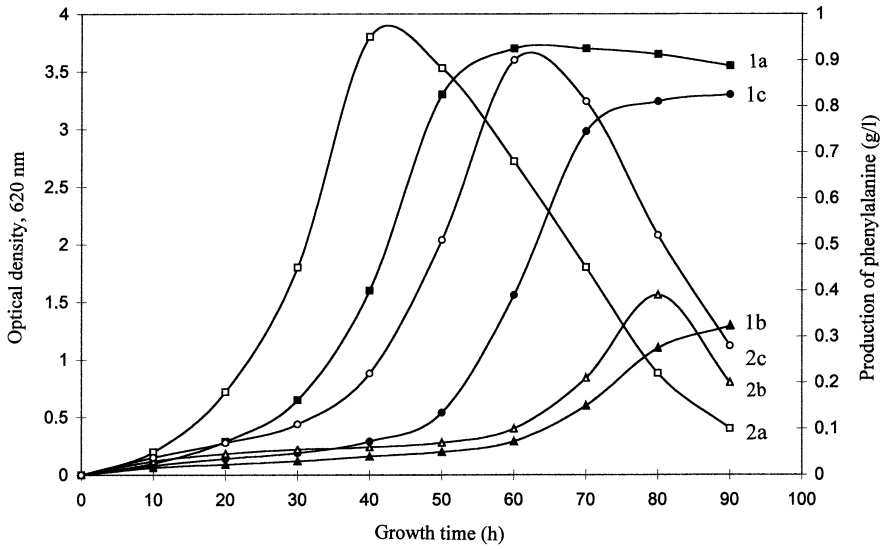


Figure 3: Growth dynamics of *B. methylicum* (1a, 2a, 3a) and production of phenylalanine (1b, 2b, 3b) on media M9 with various isotopic content: 1a, 1b – non-adapted bacterium on protonated medium (Table 1, expt. 1); 2a, 2b – non-adapted bacterium on maximally deuterated medium (Table 1, expt. 10); 3a, 3b – adapted bacterium on maximally deuterated medium (Table 1, expt. 10')

The general feature of phenylalanine biosynthesis in $H_2O/^2H_2O$-media was increase of its production at early exponential phase of growth when outputs of a microbial biomass were insignificant (Figure 3). In all the experiments it was observed that there was a decrease in phenylalanine accumulation in growth media at the late exponential phase of growth. Microscopic research of growing population of microorganisms showed that the character of phenylalanine accumulation in growth media did not correlate with morphological changes at various stages of the cellular growth. Most likely that phenylalanine, accumulated in growth media, inhibited enzymes of its biosynthetic pathways, or it later may be transformed into intermediate compounds of its biosynthesis, e.g. phenylpyruvate [27]. It is necessary to notice, that phenylalanine is synthesised in cells of microorganisms from prephenic acid, which through a formation stage of phenylpiruvate turns into phenylalanine under the influence of cellular transaminases. However, phenylalanine was not the only product of biosynthesis; other metabolically related amino acids

(alanine, valine, and leucine/isoleucine) were also produced and accumulated into growth media in amounts of 5–6 μmol in addition to phenylalanine. This fact required isolation of [^{2}H]phenylalanine from growth medium, which was carried out by extraction of lyophilized LC with iso-PrOH and the subsequent crystallization in EtOH. Analytical separation of [^{2}H]phenylalanine and metabolically related [^{2}H]amino acids was performed using a reversed-phase HPLC method on Separon SGX C_{18} Column, developed for methyl esters of N-DNS-[^{2}H]amino acids with chromatographic purity of 96–98 % and yield of 67–89 %.

With increasing ^2H_2O content in growth media, the levels of deuterium enrichment in exogenous [^{2}H]amino acids (phenylalanine, alanine, valine, and leucine/isoleucine), secreted by *B. methylicum*, were varied proportionally. The similar result on proportional specific increase of levels of deuterium enrichment into [^{2}H]phenylalanine and other metabolically related [^{2}H]amino acids was observed in all isotopic experiments where used increasing concentration ^2H_2O in growth media (Table 2). Predictably, enrichment levels of [^{2}H]phenylalanine related to the family of aromatic amino acids synthesised from shikimic acid and metabolically related [^{2}H]amino acids of pyruvic acid family – alanine, valine and leucine at identical ^2H_2O concentration in growth media are correlated among themselves. Such result is fixed in all isotope experiments with ^2H_2O (Table 2). Unlike [^{2}H]phenylalanine, deuterium enrichment levels in accompanying [^{2}H]amino acids – Ala, Val and Leu/Ile keep a stable constancy within a wide interval of ^2H_2O concentration: from 49 % (v/v) to 98 % (v/v) ^2H_2O (Table 2). Summarizing these data, it is possible to draw a conclusion on preservation of minor pathways of the metabolism connected with biosynthesis of leucine and metabolic related amino acids of pyruvic acid family – alanine and valine, which enrichment levels were in correlation within identical concentration of H_2O in growth media (phenylalanine is related to the family of aromatic amino acids synthesized from shikimic acid). Since leucine was added into growth media in protonated form, another explanation of this effect, taking into consideration the various biosynthetic pathways of Leu and Ileu (Ileu belongs to the family of aspartic acid, while Leu belongs to the pyruvic acid family), could be cell assimilation of protonated leucine from growth media. Since Leu and Ileu could not be clearly estimated by EI MS method, nothing could be said about possible biosynthesis of [^{2}H]isoleucine. Evidently, higher levels of deuterium enrichment can be achieved by replacement of protonated leucine on its deuterated analogue, which may be isolated from hydrolysates of deuterated biomass of this methylotrophic bacterium.

Table 2: Effect of deuterium enrichment levels (atom.%) in the molecules of [^{2}H]amino acids excreted by *B. methylicum**

[^{2}H]amino acid	Concentration of ^2H_2O in growth media, % (v/v)**			
	24,5	49,0	73,5	98,0
Alanine	24,0±0,70	50,0±0,89	50,0±0,83	50,0±1,13
Valine	20,0±0,72	50,0±0,88	50,0±0,72	62,5±1,40
Leucine/isoleucine	20,0±0,90	50,0±1,38	50,0±1,37	50,0±1,25
Phenylalanine	17,0±1,13	27,5±0,88	50,0±1,12	75,0±1,40

Notes:

* At calculation of enrichment levels protons (deuterons) at COOH- and NH_2-groups of amino acids were not considered because of dissociation in H_2O (^2H_2O).

** *The data on enrichment levels described bacteria grown on minimal growth media M9 containing 2 % (v/v) [U-^{2}H]MeOH and specified amounts (%, v/v) ^2H_2O.*

It should be noted that the yields of biomass on deuterated growth media were varried 85-90 % for different taxonomic groups of microorganisms. All adapted microorganisms had a slightly reduced levels of microbial biomass accumulation and increased cell generation times on maximal deuterated media.

Adaptation to deuterium the chemoheterotrophic bacterium *B. subtillis*

The result obtained in experiments on the adaptation of methylotrophic bacterium *B. methylicum* to ^2H_2O allowed to use hydrolysates of biomass of this bacterium obtained in the

process of multi-stage adaptation to ^2H_2O, as a source of deuterated growth substrates for the growing of the chemoheterotrophic bacterium *B. subtillis* and the photoorganotrophic 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 % [28]. While developing the composition of growth media on the basis of deutereted biomass of methylotrophic bacteria *B. methylicum* 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 [29]. To provide high outputs of these compounds and minimize the isotopic exchange ($^1H–^2H$) in amino acid residues of protein molecules, the biomass was hydrolyzed by autoclaving in 0,5 N ^2HCl (in ^2H_2O) and used for the growing of the chemoheterotrophic bacterium *B. subtillis* and the photoorganoheterotrophic halobacterium *H. halobium*.

The methylotrophic hydrolysate, obtained on the maximally deuterated medium M9 with 98 % (v/v) ^2H_2O and 2 % (v/v) [^{2}H]methanol, contains 15 identified amino acids (except for proline detected at λ = 440 nm) with tyrosine and histidine content per 1 gram of dry methylotrophic hydrolysate 1,82 % and 3,2 % (w/w), thereby satisfying the auxotrophic requirements of the inosine producer strain for these amino acids (Table 3). The content of other amino acids in the hydrolysate is also comparable with the needs of the strain in sources of carbon and amine nitrogen. The indicator determining the high efficiency of deuterium incorporation into the synthesized product is high levels of deuterium enrichment of amino acid molecules, varied from 50 atom% ^{2}H for leucine/isoleucine to 98,5 atom% ^{2}H for alanine (Table 3).

Table 3: Amino acid composition of hydrolyzed biomass of the facultative methylotrophic bacterium *B. methylicum* obtained on maximally deuterated M9 medium with 98 % (v/v) ^2H_2O and 2 % (v/v) [^{2}H]methanol and levels of deuterium enrichment*

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***
Glycine	9,55	2	92,5±1,86
Alanine	13,30	4	98,5±1,96
Valine	4,21	4	52,2±1,60
Leucine	8,52	5	50,0±1,52
Isoleucine	4,01	5	50,0±1,55
Phenylalanine	3,89	8	96,0±1,85
Tyrosine	2,10	7	95,5±1,82
Serine	3,60	3	86,7±1,55
Threonine	4,89	–	–
Methionine	2,62	–	–
Asparagine	10,02	2	68,5±1,62
Glutamic acid	10,31	4	70,0±1,65
Lysine	3,53	5	59,0±1,60
Arginine	4,65	–	–
Histidine	3,98	–	–

Notes: *The data were obtained for methyl esters of N-5-dimethylamino (naphthalene)-1-sulfonyl (dansyl) chloride amino acid derivatives.

** At calculation the level of deuterium enrichment, the protons (deuterons) at COOH- and NH_2-groups of amino acid molecules were not taken into account because of the dissociation in $H_2O/^2H_2O$.

*** A dash denotes the absence of data.

Taking into account the pathways of assimilation of carbon substrates, the adaptation of chemoheterotrophic bacterium *B. subtilis* was carried out via plating of initial cells to separate colonies on solid 2 % (w/v) agarose HM1 media based on 99,9 atom% ^2H_2O and deuterated hydrolyzate biomass of B. methylicum, with the following subsequent selection of the colonies resistant to ^2H_2O. On contrary to ^2H_2O, ^2H-substrates in composition of deuterated biomass hydrolyzate had no significant negative effect on the growth parameters of the studied microorganisms. The growth and biosynthetic characteristics of inosine-producing strain *B. subtilis* were studied on protonated yeast PVC medium with H_2O and 2 % (w/v) yeast PVC and on HW medium with 89 % (v/v) ^2H_2O and 2 % (w/w) hydrolysate of deuterated biomass of *B. methylicum* (Figure 4). Experiments demonstrated a certain correlation between the changes of growth dynamics of *B. subtilis* (Figure 4, curves *1*, *1*'), output of inosine (Figure 4, curves *2*, *2*'), and glucose assimilation (Figure 4, curves *3*, *3*'). The maximal output of inosine (17 g/l) was observed on protonated PVC medium at a glucose assimilation rate 10 g/l (Figure 4, curve *2*). The output of inosine in the HW medium decreased in 4,4-fold, reaching 3,9 g/l (Figure 4, curve *2*'), and the level of glucose assimilation – 4-fold, as testified by the remaining 40 g/l non-assimilated glucose in CL (Figure 4, curve *3*'). The experimental data demonstrate that glucose is less efficiently assimilated during growth in the HW medium as compared to the control conditions in H_2O.

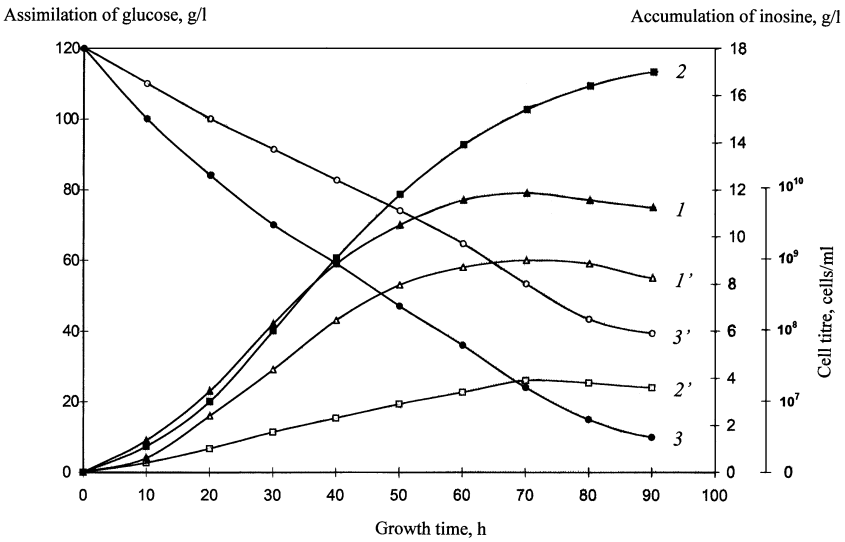


Figure 4: Growth dynamics of *B. subtilis* (*1*, *1*') (cells/ml), inosine accumulation in LC (*2*, *2*') (g/l), and glucose assimilation (*3*, *3*') (g/l) under different experimental conditions: (*1*–*3*) – protonated yeast PVC medium; (*1*'–*3*') – HW medium with 2% (w/v) hydrolysate of deuterated biomass of *B. methylicum*.

The isolation of inosine from the CL consisted in low-temperature precipitation of high molecular weight impurities with organic solvents (acetone and methanol), adsorption/desorption on the surface of activated carbon, extraction of the end product, crystallization, and ion exchange chromatography. The proteins and polysaccharides were removed from the CL by precipitation with acetone at 4 ^{0}C with subsequent adsorption/desorbtion of total ribonucleosides on activated carbon. The desorbed ribonucleosides were extracted from the reacted solid phase by eluting with EtOH-NH_3-solution at 60 ^{0}C; inosine – by extracting with 0,3 M ammonium–formate buffer (pH = 8,9) and subsequent crystallization in 80% (v/v) ethanol. The final purification consisted in column ion exchange chromatography on AG50WX 4 cation exchange resin equilibrated with 0,3 M ammonium–formate buffer containing 0,045 M NH_4Cl with collection of fractions at R_f = 0,5. The curves *1*–*3* in Figure 5 show UV-absorption spectra of inosine isolated from the CL at various stages of isolation procedure. The presence of major absorbance band I, corresponding to natural inosine (λ_{max} = 249 nm, ε_{249} = 7100 $M^{-1}cm^{-1}$), and the absence of secondary metabolites II and III in the analyzed sample (Figure 5, curve *3*), demonstrates the homogeneity of the isolated product and the efficiency of the isolation method.

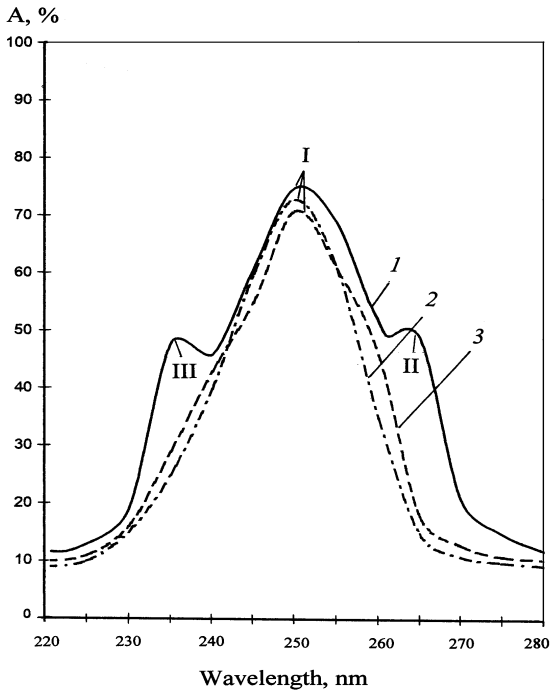


Figure 5: UV-absorption spectra of inosine (0,1 N HCl): (*1*) – initial LC after the growth of *B. subtilis* on HW medium; (*2*) – natural inosine, and (*3*) – inosine extracted from the LC. Natural inosine (*2*) was used as a control: (I) – inosine, (II, III) – secondary metabolites.

The level of deuterium enrichment of [^{2}H]inosine was determined by FAB mass spectrometry, the high sensitivity of which allows to detect 10^{-8} to 10^{-10} moles of a substance in a sample. The formation of a molecular ion peak for inosine in FAB mass spectrometry was accompanied by the migration of H^+. Biosynthetically ^{2}H-labeled inosine**,** which FAB mass-spectrum represented in Figure 6*b* regarding the control (natural protonated inosine, Figure 6*a*), represented a mixture of isotope-substituted molecules with different numbers of hydrogen atoms replaced by deuterium. Correspondingly, the molecular ion peak of inosine $[M+H]^+$, was polymorphically splintered into individual clusters with admixtures of molecules with statistical set of mass numbers *m*/*z* and different contributions to the total level of deuterium enrichment of the molecule. It was calculated according to the most intensive molecular ion peak (the peak with the largest contribution to the level of deuterium enrichment) recorded by a mass spectrometer under the same experimental conditions. These conditions are satisfied the most intensive molecular ion peak $[M+H]^+$ at *m*/*z* 274 with 38 % (instead of $[M+H]^+$ at *m*/*z* 269 with 42 % under the control conditions; Figure 6*a*). That result corresponds to five deuterium atoms incorporated into the inosine molecule (Figure 6b). The molecular ion peak of inosine also contained less intensive peaks with admixtures of molecules containing four (*m*/*z* 273, 20 %), five (*m*/*z* 274, 38 %), six (*m*/*z* 275, 28 %), and seven (*m*/*z* 276, 14 %) deuterium atoms (Table 4).

Table 4: Values of peaks $[M+H]^+$ in the FAB mass spectra and levels of deuterium enrichment of inosine isolated from HW-medium

Value of peak $[M+H]^+$	Contribution to the level of deuterium enrichment, mol.%	The number of deuterium atoms	Level of deuterium enrichment of molecules, % of the total number of hydrogen atoms*
273	20	4	20,0±0,60
274	38	5	62,5±1,80
275	28	6	72,5±1,96
276	14	7	87,5±2,98

Notes: *At calculation of the level of deuterium enrichment, the protons(deuterons) at the hydroxyl (OH^-) and imidazole protons at NH^+ heteroatoms were not taken into account because of keto–enol tautomerism in $H_2O/^2H_2O$.

Taking into account the contribution of the molecular ion peaks $[M]^+$, the total level of deuterium enrichment (TLDE) of the inosine molecule calculated using the below equation was 65,5 % of the total number of hydrogen atoms in the carbon backbone of the molecule:

$$TLDE = \frac{[M]^+_{r1} \cdot C_2 + [M]^+_{r2} \cdot C_2 + ... + [M]^+_{rn} \cdot C_n}{\sum C_n}$$

where $[M]^+_r$ - the values of the molecular ion peaks of inosine; C_n - the contribution of the molecular ion peaks to TLDE (mol %).

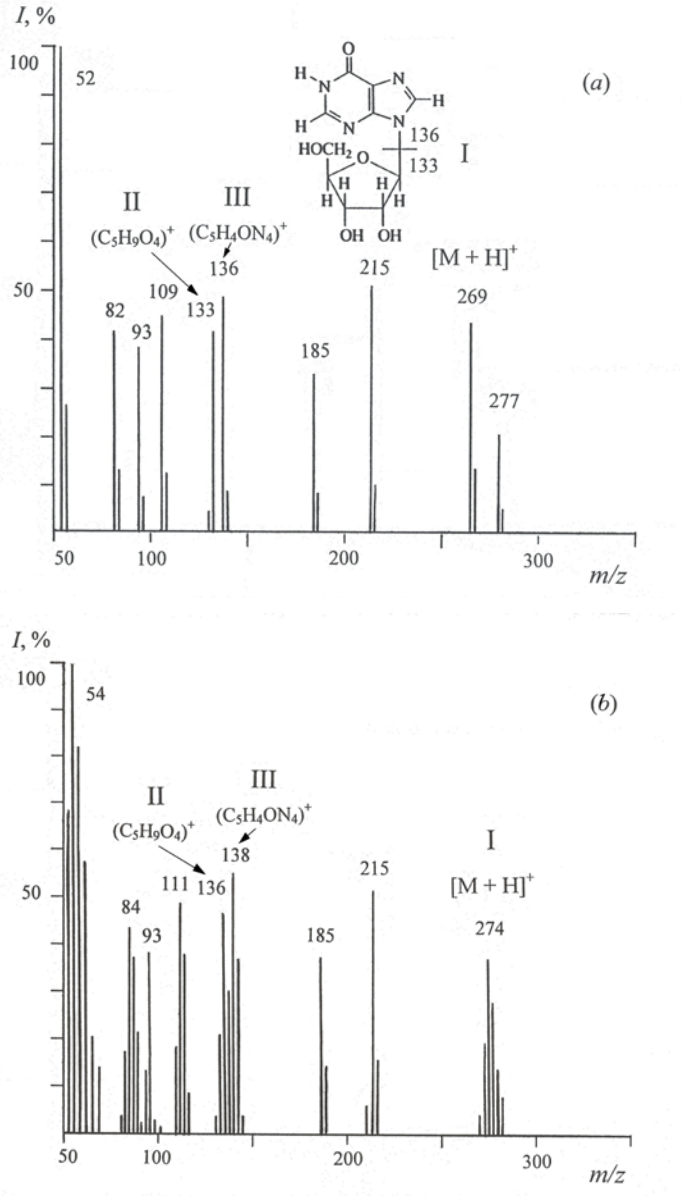


Figure 6: FAB mass spectra of inosine (glycerol as a matrix) under different experimental conditions: (*a*) – natural inosine; (*b*) – [^{2}H]inosine isolated from HW medium (scanning interval at *m*/*z* 50–350; major peaks with a relative intensity of 100 % at *m*/*z* 52 and *m*/*z* 54; ionization conditions: cesium source; accelerating voltage, 5 kV; ion current, 0,6–0,8 mA; resolution, 7500 arbitrary units): *I* – relative intensity of peaks (%); (I) – inosine; (II) – ribose fragment; (III) – hypoxanthine fragment.

The fragmentation of the inosine molecule, shown in Figure 7, gives more precise information on the deuterium distribution in the molecule. The FAB fragmentation pathways of the inosine

molecule (I) lead to formation of ribose $(C_5H_9O_4)^+$ fragment (II) at *m*/*z* 133 and hypoxanthine $(C_5H_4ON_4)^+$ fragment (III) at *m*/*z* 136 (their fragmentation is accompanied by the migration of H^+), which in turn, later disintegrated into several low-molecular-weight splinter fragments at *m*/*z* 109, 108, 82, 81, and 54 due to HCN and CO elimination from hypoxanthine (Figure 7). Consequently, the presence of two "heavy" fragments of ribose II $(C_5H_9O_4)^+$ at *m*/*z* 136 (46 %) (instead of *m*/*z* 133 (41 %) in the control) and hypoxanthine III $(C_5H_4ON_4)^+$ at *m*/*z* 138 (55 %) (instead of *m*/*z* 136 (48 %) in the control), as well as the peaks of low molecular weight splinter fragments formed from FAB-decomposition of hypoxanthine fragment at *m*/*z* 111 (49 %) (instead of *m*/*z* 109 (45 %) in the control) and *m*/*z* 84 (43 %) (instead of 82 (41 %) in the control) suggests that three deuterium atoms are incorporated into the ribose residue, and two other deuterium atoms – into the hypoxanthine residue of the inosine molecule (Figure 7). Such selective character of the deuterium inclusion into the inosine molecule on specific locations of the molecule was confirmed by the presence of deuterium in the smaller fission fragments.

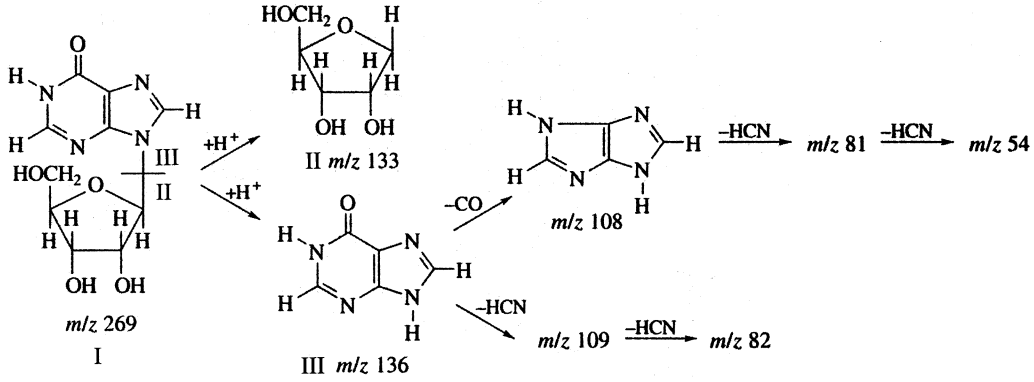


Figure 7: The fragmentation pathways of the inosine molecule leading to formation of smaller fragments by the FAB-method

The metabolic pathways of assimilation of glucose under aerobic conditions by chemoheterotrophic bacteria include the Embden-Meyerhof pathway; the anaerobic glycolysis is not widespred in this type of bacteria. When analyzing the level of deuterium enrichment of the inosine molecule we took into account the fact that the character of deuterium incorporation into the molecule is determined by the pathways of carbon assimilation (both glucose and amino acids). The carbon source was glucose as a main substrate and a mixture of deuterated amino acids from deuterated hydrolysate of methylotrophic bacterium *B. methylicum* as a source of deuterated substrates and amine nitrogen. Since the protons (deuterons) at positions of the ribose residue in the inosine molecule could have been originated from glucose, the character of deuterium inclusion into the ribose residue is mainly determined by the assimilation of glucose by glycolysis, associated with the Embden-Meyerhof pathway. The decomposition of glucose into two molecules of pyruvate is carried out in 10 stages, the first five of which are a preparatory stage and the next 5 – the stage interfaced with the formation of ATP. During the glycolysis glucose is phosphorylated at hydroxyl group at the sixth carbon atom (C-6), forming glucose-6-phosphate (step 1). Glucose 6-phosphate is then isomerized to fructose-6-phosphate (step 2), which is phosphorylated at the hydroxyl group at the first carbon atom, with the formation of fructose 1,6-bisphosphate (step 3). During both of these reactions of phosphorylation as a donor of phosphoryl group acts ATP. Next fructose-1,6-diphosphate is split into two three-carbon molecules – glyceroldehyde 3-phosphate and dihydroxyacetone phosphate (step 4), which in the result by means of several enzymatic reactions (5–10) is converted to piruvate.

The overall equation of glycolysis:

$$Glucose + 2NAD^+ + 2ADP + 2P_i \rightarrow 2\ piruvate + 2NADH + 2H^+ + 2ATP + 2H_2O,$$

Most chemoheterotrophic bacteria from I group can grow under anaerobic conditions via fermentation of sugars (glycolysis), the main products of which are 2,3-butanediol, glycerol and

CO_2; besides are formed minor amounts of formed lactic acid and ethanol. This type of fermentation can be represented as follows:

3 mol. glucose → 2,3-butanediol + 2 mol. glycerol + 4 mol. CO_2

Glucose is initially split via the Embden-Meyerhof pathway to glyceroldehyde 3-phosphate; after that there is branching pathway. Some part of glyceroldehyde 3-phosphate is converted to dihydroxyacetone phosphate, while another part – to pyruvate, which is formed from 2,3-butanediol and CO_2. Formation of 2,3-butanediol from pyruvate leads to the re-oxidation of the NAD·H formed during the conversion of glyceroldehyde 3-phosphate to pyruvate:

2 mol. glyceroldehyde 3-phosphate + 2 mol. NAD^+ + 4 mol. ADP + 2 mol. P → 2 mol. pyruvate + 4 mol. ATP + 2 mol. NAD·H + 2 mol. H^+,

2 mol. pyruvate + NAD·H + H^+ → 2 mol. CO_2 + 2,3-butanediol + NAD^+.

The redox equilibrium is maintained by the concomitant restoration of glyceroldehyde 3-phosphate to glycerol:

Glyceroldehyde 3-phosphate + NAD·H + H^+ → Glycerol + P + NAD^+.

Since glucose in our experiments was used in a protonated form, its contribution to the level of deuterium enrichment of the ribose residue was neglected. However, as the investigation of deuterium incorporation into the molecule by FAB method showed that deuterium was incorporated into the ribose residue of the inosine molecule owing to reaction of enzymatic izomerization of glucose. The numerous isotopic $^1H–^2H$ exchange processes could also have led to specific incorporation of deuterium atoms at certain positions in the inosine molecule. Such accessible positions in the inosine molecule are hydroxyl (OH^-)- and imidazole protons at NH^+ heteroatoms, which can be easily exchanged on deuterium in ^2H_2O *via* keto–enol tautomerism. Three non-exchangeable deuterium atoms in the ribose residue of inosine are synthesized *de novo* and could have been originated via enzymatic assimilation of glucose by the cell, while two other deuterium atoms at C2,C8-positions in the hypoxanthine residue could be synthesized *de novo* at the expense of [^{2}H]amino acids, primarily glutamine and glycine, that originated from the deuterated hydrolysate of methylotrophic bacterium *B. methylicum* obtained on 98 % of ^2H_2O medium. In general, our studies confirmed this scheme. However, it should be noted that auxotrophy of this strain in tyrosine, histidine, adenine and uracil presupposes some other inosine biosynthesis pathway, different that indicated in Fig. 8. The level of deuterium enrichment of the inosine molecule is determined by isotopic purity of ^2H_2O and deuterated substrates and, therefore, for the total administration of the deuterium label into the inosine molecule instead of protonated glucoce it must be used its deuterated analogue. Deuterated glucose may be isolated in gram-scale quntities from deuterated biomass of the methylotrophic bacterium *B. methylicum*.

Our experiments demonstrated that chemo-heterotrophic metabolism does not undergo significant changes in ^2H_2O. This testifies about a phenotypic nature of adaptation to ^2H_2O phenomenon as the adapted cells eventually return back to the normal growth after some lag-period after their replacement back onto H_2O-medium. However, the effect of reversion of growth on $H_2O/^2H_2O$ media does not exclude an opportunity that a certain genotype determines the manifestation of the same phenotypic attribute in ^2H_2O-media with high deuterium content. At placing a cell onto ^2H_2O-media lacking protons, not only ^2H_2O is removed from a cell due to isotopic ($^1H–^2H$) exchange, but also there are occurred a rapid isotopic ($^1H–^2H$) 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 ($^1H–^2H$) exchange and, thereof only molecules with bonds such as C–^{2}H can be synthesized de novo (Mosin et al., 1996b; Mosin & Ignatov, 2012a). Thus, the most sensitive to replacement of H on ^{2}H 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 ^2H_2O as adaptation to the nonspecific factor affecting simultaneously the functional condition of several numbers of cellular systems: metabolism, ways of assimilation of carbon substrates, biosynthetic processes, and transport function, structure and functions of macromolecules.

Adaptation to deuterium the microalgae *C. vulgaris*

For adaptation of microalgae *C. vulgaris* was used Tamiya liquid mineral medium containing 25; 50; 75 and 98 % (v/v) ^2H_2O. The levels of deuterium enrichment of carotenoids were In the case of C. vulgaris and H. halobium used fluorescent illumination, as both microorganisms grown in the presence of light. Individual colonies of cells of these microorganisms resistant to ^2H_2O, allocated by selection were grown on liquid growth media of the same composition with 99,9 atom% ^2H_2O for producing the deuterated biomass.

Adaptation to deuterium the photoorganotrophic halobacterium *H. halobium*

The cell membrane of extreme aerobic photo-organotrophic halobacterium *Halobacterium halobium* contains a chromoprotein trans-membrane protein - bacteriorhodopsin (BR) with the molecular weight ~26,5 kDa, determining the purple-red culour of halophilic bacteria. BR contains as chromophore group an equimolar mixture of 13-*cis*- and 13-*trans*-retinol C20 carotenoid, bound by aldemine bond schiff base (as in the visual animal pigments) with Lys-216 residue of the protein. In halobacteria BR functions as a light-driven transmembrane proton pump pumping a proton accros the membrane. Along with the BR the cell membrane of halobacteria contains a small amount of other related carotenoid pigments, the main of which bakterioruberin determining the stability of halobacteria to solar radiation.

The adaptation of photo-organotrophic halobacterium *Halobacterium halobium* was carried out via plating of initial cells to separate colonies on solid 2 % (w/v) agarose HM2 media based on 99,9 atom% ^2H_2O and deuterated hydrolyzate biomass of *B. methylicum*, with the following subsequent selection of the colonies resistant to ^2H_2O. The growing of halobacteria was carried out under illumination by light fluorescent lamps LDS-40-2 (40 W) with monochromatic light with λ = 560 nm for 4–5 days at 35 ^{0}C as swoun in Figure 8. While growing of *H. halobium* on HM2 growth medium cells synthesized the purple carotenoid pigment, identified as a native BR on the the spectral ratio of protein and chromophore fragments in the molecule (D_{280}/D_{568} = 1,5:1,0). The growth of this bacterium on ^2H_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 the halobacterium on deuterated growth medium with 2 % (w/v) of deuterated biomass hydrolyzate of *B. methylicum*, cell disintegration and lysis; isolation of purple membrane (PM) fraction; purification of PM from the low and high-molecular weight impurities, cellular RNA, carotenoids and phospholipids; solubilization of PM in 0,5 % (w/v) solution of ionic detergent SDS–Na to form a microemulsion; fractionation of solubilized BR by MeOH; gel permeation chromatography (GPC) on Sephadex G-200 and electrophoresis in 12,5 % (w/v) PAAG in 0,1 % (w/v) SDS -Na.

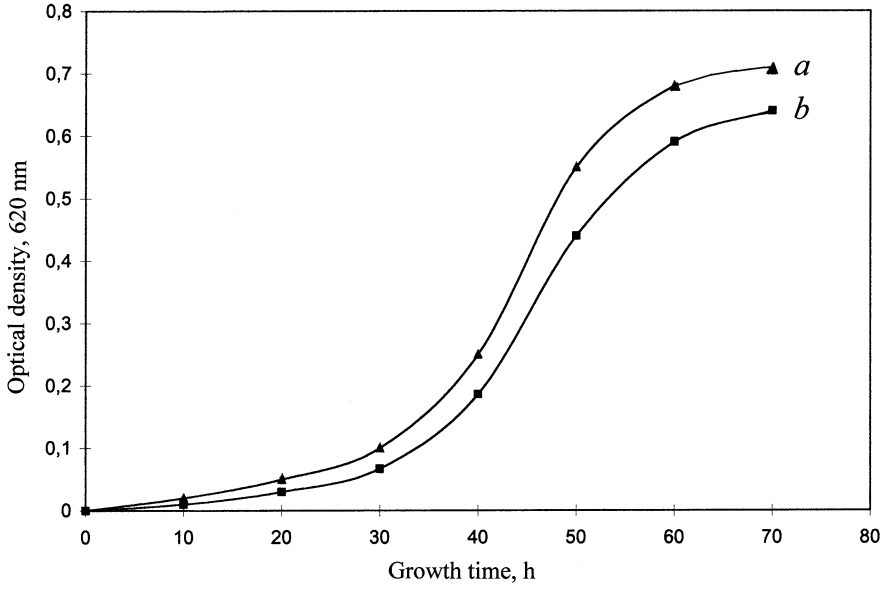


Figure 8: Growth dynamics of *H. halobium* under various experimental conditions: *a*) – HW2-medium; *b*) – peptone medium. Growing conditions: the incubation period: 4–5 days, temperature: 35 ^{0}C, illumination under monochrome light at λ = 560 nm

In an attempt to remove a large fraction of the carotenoids and phospholipids from the membrane by column GPC, PM fraction was washed by 50 % (v/v) of EtOH before stabilization by SDS-Na. Removing of carotenoids, consisting in repeated treatment of PM with 50 % (v/v) EtOH at 0 ^{0}C, was a routine but necessary step, in spite of the significant loss of the chromoprotein. It was used five treatments by 50 % (v/v) EtOH to obtain the absorption spectrum of PM suspension purified from carotenoids (4) and (5) (degree of chromatographic purity of 80-85 %), as shown in Figure 9 at various processing stages (*b*) and (*c*) relative to the native BR (a). Figure 9 shows a dark-adapted absorption maximum at λ = 548 nm. Formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption spectrum of PM (Figure 9*c*) - the main bandwith (1) with the absorption maximum at λ = 568 nm caused by the light isomerization of the chromophore by the C13=C14 bond is determined by the presence of 13-*trans*-retinal residue in BR_{568}; additional low-intensity bandwith (2) at λ = 412 nm characterizes a minor impurity of a spectral form of *meta*-bacteriorhodopsin M_{412} (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwith (3) with λ = 280 nm is determined by the absorption of aromatic amino acids in the polypeptide chain of the protein (for native BR D_{280}/D_{568} = 1,5:1,0). Upon the absorption of light, the maximum absorbance of PM shifts to λ = 556 nm with 6-8 % increase in extinction. The 280/568 nm absorbance ratio of BR is directly related to the ratio of total protein (native BR) and is a convenient indicator for BR stability and integrity. Identical absorbance ratios are monitored using the conventional optics on a Beckman DU-6 spectrophotometer (“Beckman Coulter”, USA) for detergent-solubilized BR or purified BR-solubilized in detergent.

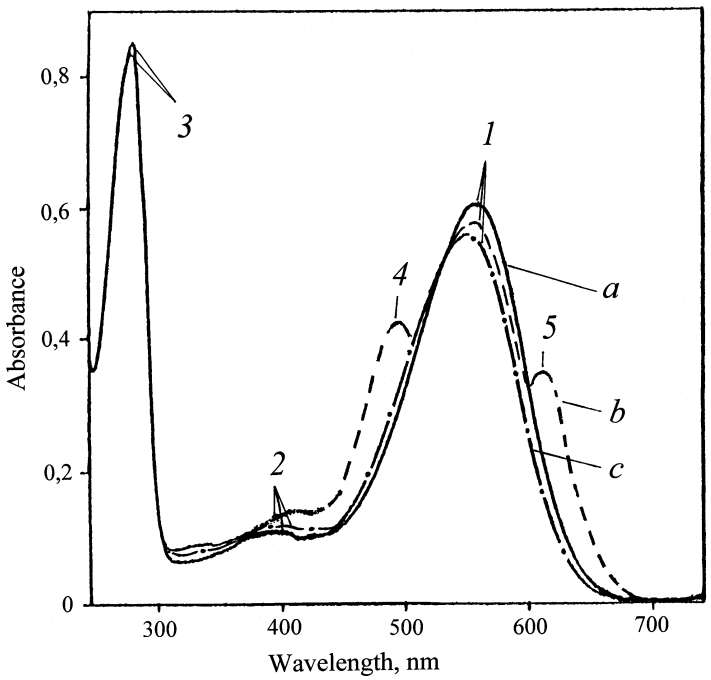


Figure 9: The absorption spectra of PM (50 % (v/v) EtOH) at various stages of processing: (*a*) – natural BR; (*b*) – PM after intermediate treatment; (*c*) – PM purified from carotenoids. The bandwith (1) is the spectral form of BR_{568}, (2) – impurity of spectral form of *meta*-bacteriorhodopsin M_{412}, (3) – the total absorption bandwith of aromatic amino acids, (4) and (5) – extraneous carotenoids. The native BR A was used as a control.

The final stage of purification involved the crystallization of the solubilized in 0,5 % (w/v) SDS-Na solution protein by MeOH and further separation of the protein from low-molecular-weight impurities by GPC. For this purpose the fractions containing BR were passed twice through a column with dextran Sephadex G-200 balanced with 0.09 M Tris buffer (pH = 8,35) containing 0,1 % (w/v) SDS-Na and 2,5 mM EDTA.

The homogeneity of isolated BR satisfies to the requirements for reconstruction of native membranes, and was confirmed by electrophoresis in 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na and *in vitro* regeneration of AP with 13-*trans*-retinal. The degree of regeneration of PM was determined by the ratio: $D_{nat.280} \cdot D_{nat.568} / D_{reg.280} \cdot D_{reg.568}$ (D_{280} and D_{568} – the absorbance of a suspension of native and regenerated PM at λ = 280 and λ = 568 nm) was 65 mol.%. Output of crystalline protein makes up approximately 5 mg. 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% ^2H.

Discussion

Our studies indicated that the ability of adaptation to ^2H_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 ^2H_2O, contrary to blue-green algae, which, being eukaryotes, are the more difficult adapted to ^2H_2O and, therefore, 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 ^2H_2O, because the reason of inhibition of cell growth and cell death can be changes of

the parity ratio of synthesized metabolites in ^2H_2O-media: amino acids, proteins and carbohydrates. It is noted that adaptation to ^2H_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 ^2H_2O of different vital systems is different. 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. 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 green algae *C. vulgaris*. Cells grown on ^2H_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 (Figure 10). 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 [30]. 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 ^2H_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 ^2H_2O prokaryotic and eukaryotic cells.

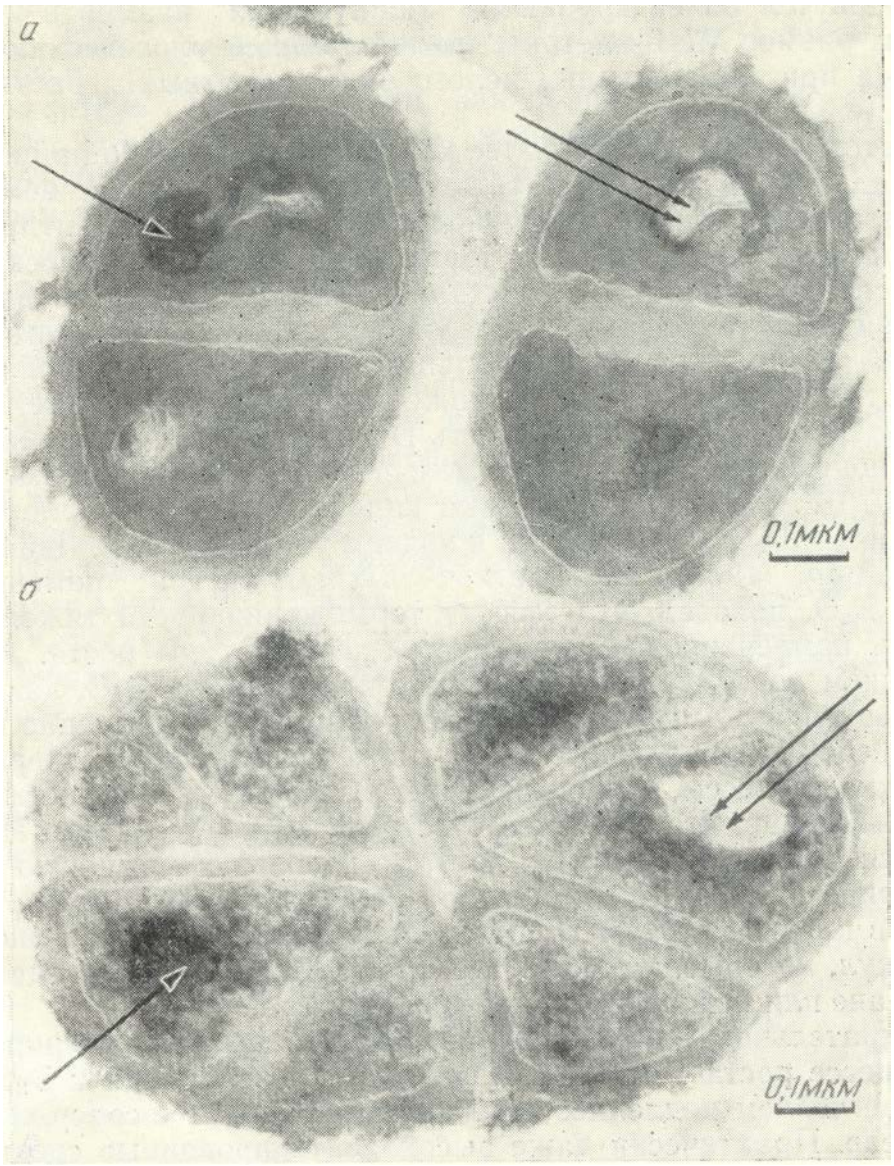


Figure 10: Electron micrographs of *Micrococcus lysodeikticus* cells obtained by SEM method: *a*) – protonated cells obtained on H_2O-medium; *b*) – deuterated cells obtained on ^2H_2O-medium. The arrows indicate the tightly-packed portions of the membranes

Our data generally confirm a stable notion that adaptation to ^2H_2O is a phenotypic phenomenon as the adapted cells eventually return back to the normal growth after some lag-period after their replacement back onto H_2O-medium. However, the effect of reversion of growth on $H_2O/^2H_2O$ media does not exclude an opportunity that a certain genotype determines the manifistation of the same phenotypic attribute in ^2H_2O-media with high deuterium content. At placing a cell onto ^2H_2O-media lacking protons, not only ^2H_2O is removed from a cell due to isotopic ($^1H-^2H$) exchange, but also there are occurred a rapid isotopic ($^1H-^2H$) 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 ($^1H-^2H$) exchange and, thereof only molecules with bonds such as C–^2H can be synthesized de novo. 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 $^1H(^2H)$ atoms and adjacent electronegative O, C, N, S- heteroatoms in the molecules, acting as acceptors of H-bond. The hydrogen bond, based on weak electrostatic forces, donor-acceptor interactions with charge-transfer and intermolecular van der Waals forces, is of the vital importance in the chemistry

of intermolecular interactions and maintaining the spatial structure of macromolecules in aqueous solutions. Another important property is defined by the three-dimensional structure of ^2H_2O molecule having the tendency to pull together hydrophobic groups of macromolecules to minimize their disruptive effect on the hydrogen (deuterium)-bonded network in ^2H_2O. This leads to stabilization of the structure of protein and nucleic acid macromolecules in the presence of ^2H_2O. That is why, the structure of macromolecules of proteins and nucleic acids in the presence of ^2H_2O is somehow stabilized [31].

Evidently the cell implements special adaptive mechanisms promoting the functional reorganization of vital systems in ^2H_2O. Thus, for the normal synthesis and function in ^2H_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 $^2H–O$ bond compared to $^1H–O$ bond causes the differences in the kinetics of reactions in H_2O and ^2H_2O. Thus, according to the theory of a chemical bond the breaking up of covalent $^1H–C$ bonds can occur faster than $C–^2H$ bonds, the mobility of $^2H_3O^+$ ion is lower on 28,5 % than H_3O^+ ion, and O^2H^- ion is lower on 39,8 % than OH^- ion, the constant of ionization of ^2H_2O is less than that of H_2O. These chemical-physical factors lead to slowing down in the rates of enzymatic reactions in ^2H_2O [32]. However, there are also such reactions which rates in ^2H_2O are higher than in H_2O. In general these reactions are catalyzed by $^2H_3O^+$ or H_3O^+ ions or O^2H^- and OH^- ions. The substitution of ^1H with ^2H 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 ^2H_2O. It may cause disturbances in the DNA-synthesis during mitosis, leading to permanent changes on DNA structure and consequently on cell genotype [33]. Isotopic effects of deuterium, which would occur in macromolecules of even a small difference between hydrogen and deuterium, would certainly have the effect upon the structure. The sensitivity of enzyme function to the structure and the sensitivity of nucleic acid function (genetic and mitotic) would lead to a noticeable effect on the metabolic pathways and reproductive behaviour of an organism in the presence of ^2H_2O. And next, the changes in dissociation constants of DNA and protein ionizable groups when transferring the macromolecule from H_2O into ^2H_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 ^2H_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 ^2H_2O. But it has been not clearly known what occurs with the membranes – how they react to the replacement of protium to deuterium and how it concerns the survival of cells in ^2H_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.% ^2H_2O, carried out by HPLC method, revealed significant quantitative differences in the fatty acid composition compared to the control obtained in ordinary water (Figure 11a, b). Characteristically, in a deuterated sample fatty acids having retention times at 33,38; 33,74; 33,26 and 36,03 min are not detected in HPLC-chromatogram (Fig. 11b). This result is apparently due to the fact that the cell membrane is one of the first cell organelles, sensitive to the effects of ^2H_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. The similar situation was observed with the separation of other natural compounds (proteins, amino acids, carbohydrates) extracted from deutero-biomass obtained from maximally deuterated ^2H_2O-medium.

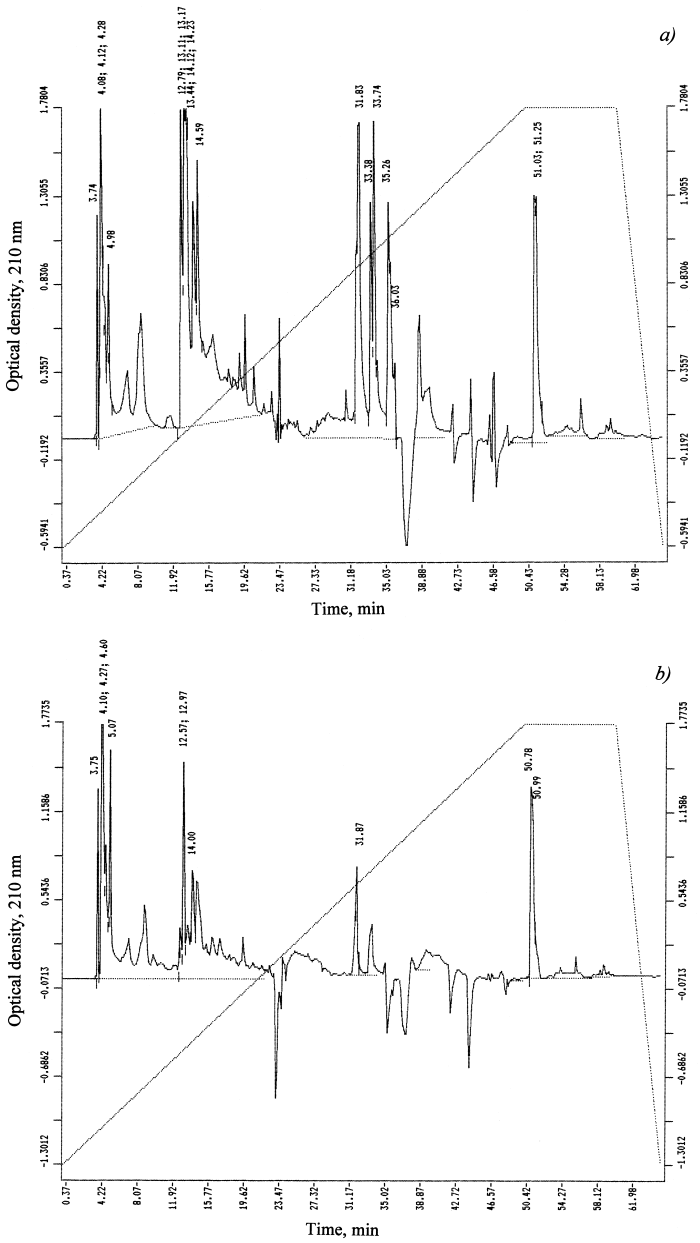


Figure 11: HPLC-chromatograms of fatty acids obtained from protonated (*a*) and deuterated (*b*) cells of *B. subtilis* on the maximally deuterated ^2H_2O-medium: Beckman Gold System (Beckman, USA) chromatograph (4,6×250 mm); stationary phase: Ultrasphere ODS, 5 μm; mobile phase: linear gradient 5 mM KH_2PO_4–acetonitrile (shown in phantom), elution rate: 0,5 ml/min, detection at λ = 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 isolated from deuterated cells of B. subtilis*, also revealed the differences in quantitative composition of amino acids synthesized in ^2H_2O-medium (Figure 12). Protein hydrolyzates contains fifteen identified amino acids (except proline, which was detected at λ = 440 nm) (Table 5). 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.*

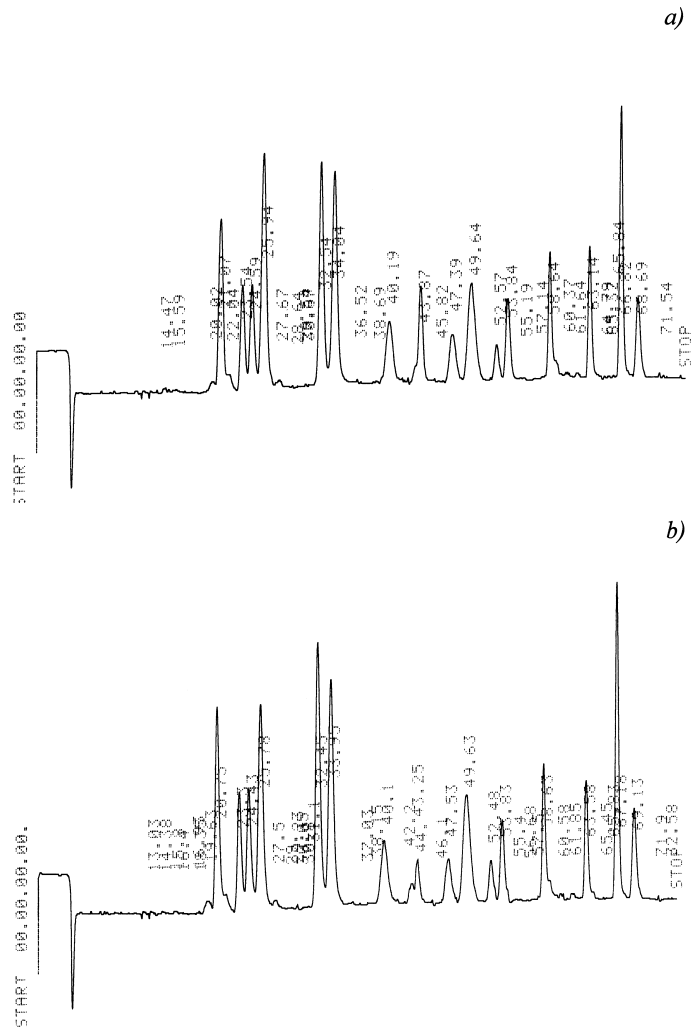


Figure 12: Ion-exchange chromatograms of amino acids obtained from hydrolizates of protonated (a) and deuterated (b) cells of *B. subtilis* on the maximally deuterated D_2O-medium: Biotronic LC-5001 (230×3,2 mm) column ("Eppendorf–Nethleler–Hinz", Germany); stationary phase: UR-30 sulfonated styrene 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).

Table 5: 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.% ^2H_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:

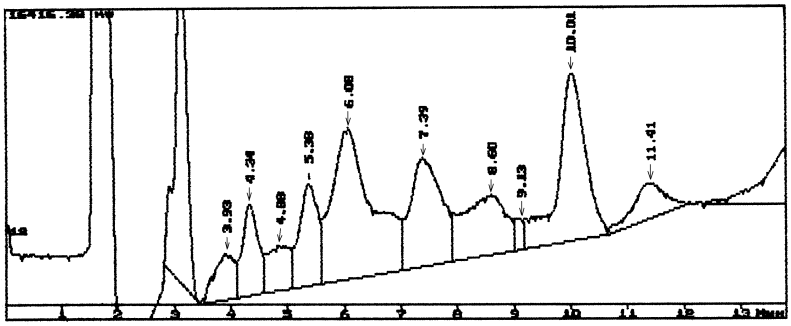
* The data obtained by mass spectrometry for the methyl esters of N-5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl) amino acid derivatives.

** 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 $H_2O/^2H_2O$

*** A dash means absence of data.

Qualitative and quantitative composition of the intracellular carbohydrates of *B. subtilis* obtained on maximally deuterated ^2H_2O-medium is shown in Table. 6 (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) (Figure 13). 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 6). The deuterium enrichment levels of carbohydrates were varied from 90,7 atom.% for arabinose to 80,6 atom.% for glucose.

a)



b)

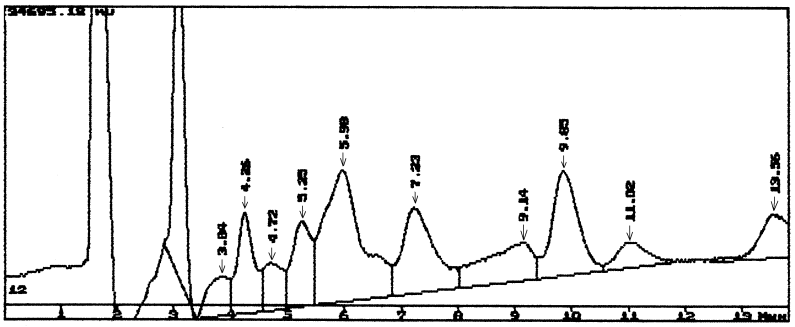


Figure 13: HPLC-chromatograms of intracellular carbohydrates obtained from protonated (a) and deuterated (b) cells of *B. subtilis* on the maximally deuterated ^2H_2O-medium: Knauer Smartline chromatograph (250×10 mm) ("Knauer", Germany); stationary phase: Ultrasorb CN; 10 μm; mobile phase: acetonitrile–water (75:25, % (w/w); the input rate: 0,6 ml/min

Table 6: 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.% ^2H_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.

Electrophoregrams of proteins isolated from hydrolysates of the microbial biomass of *B. subtilis* grown on ^2H_2O also showed the differences in the qualitative composition of total protein obtained in ^2H_2O (Figure 14).

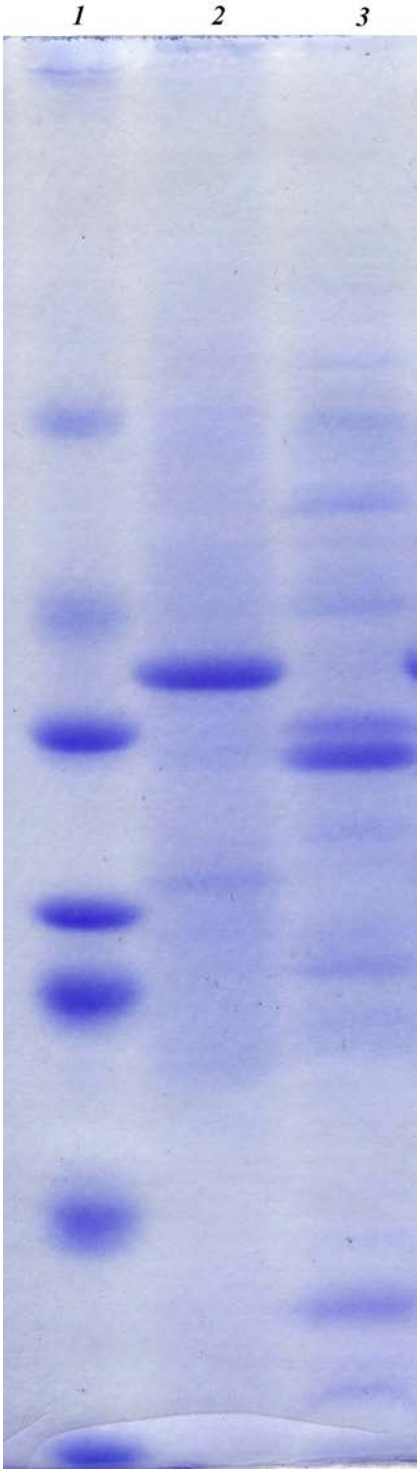


Figure 14: Electrophoregrams of proteins isolated from hydrolysates of total biomass of *B. subtilis*: *1* – a standard set of proteins; *2* – a sample obtained from protonated medium; *3* – a sample obtained from D_2O-medium

Conclusions

The experimental data demonstrated that the effects observed at the cellular growth on ^2H_2O-media 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 ^2H_2O, and with an evolutionary level of organization of investigated objects as well. The cell evidently implements the special adaptive mechanisms promoting functional reorganization of work of the vital systems in the presence of ^2H_2O. Thus, the most sensitive to replacement of ^1H on ^2H 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 $^{2}H_2O$ 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 $^{2}H_2O$ in growth media.

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УДК 579.871.08

Исследования цитологических, морфологических и физиологических изменений в клетках прокариот и эукариот при росте на тяжелой воде

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Аннотация. Исследовано биологическое влияние дейтерия на клетки различных таксономических групп прокариотических и эукариотических микроорганизмов, реализующих метилотрофный, хемогетеротрофный, фото-органотрофный и фотосинтетический способы ассимиляции углеродных субстратов (метилотрофные бактерии *Brevibacterium methylicum*, хемогетеротрофные бактерии *Bacillus subtilis*, фото-органотрофные галобактерии *Halobacterium halobium* и зеленая микроводоросль *Chlorella vulgaris*) при росте на питательных средах с тяжелой водой (^2H_2O). Для исследуемых микрооорганизмов представлены данные по росту и адаптации на питательных средах, содержащих в качестве источников дейтерированных субстратов ^2H_2O, [^{2}H]метанол и гидролизаты дейтерированного биомассы метилотрофных бактерий *B. methylicum*, полученных в условиях многоступенчатой адаптации к ^2H_2O. Приведен качественный и количественный состав внутри-и межклеточных аминокислот, белков, углеводов и жирных кислот в условиях адаптации к ^2H_2O. Показано, что эффекты, наблюдаемые при адаптации к ^2H_2O, имеют сложный многофакторный характер и связаны с цитологическими, морфологическими и физиологическими изменениями в клетке - величины лаг-периода, времени клеточной генерации, выходах биомассы, соотношении синтезированных аминокислот, белков, углеводов и липидов, а также с эволюционным уровнем организации исследуемого объекта и путями ассимиляции углерода субстратов.

Ключевые слова: дейтерий, тяжелая вода, адаптация, изотопные эффектты, бактерии, микроводоросли.

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Possible North-Eastern Connections of the R1a1-populations of Corded Ware Culture According to the Archaeologic and Paleogenetic Data

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Abstract

Our new work considers the problems of paleogenetics, archeology and antropology connected with origins of Corded Ware culture and early migration of Y-DNA R1 carriers. This work considers the Second Corded Ware Center on the Far East and Yakutia and its connection with the Eastern European one. Authors examine the hypothesis that the two Corded Ware cultures have the common source.

Keywords: R1a1, Mesolithic, Yuzni Oleni Ostrov, paleogenetics, paleolinguistics, subclades, Yakutia, Na-Dene.

Introduction

The emergence of Corded Ware culture is concerned with «the setting of agricultural and cattle breeding tribes among the local late Neolithic ones». They had similar features in forms and ornamentations of pottery, types of stone tools, funeral rites. It makes it possible to bring them together into cultural and historic area of the corded ware tribes, which occupied the huge territory in Europe» [**1, p. 35**].

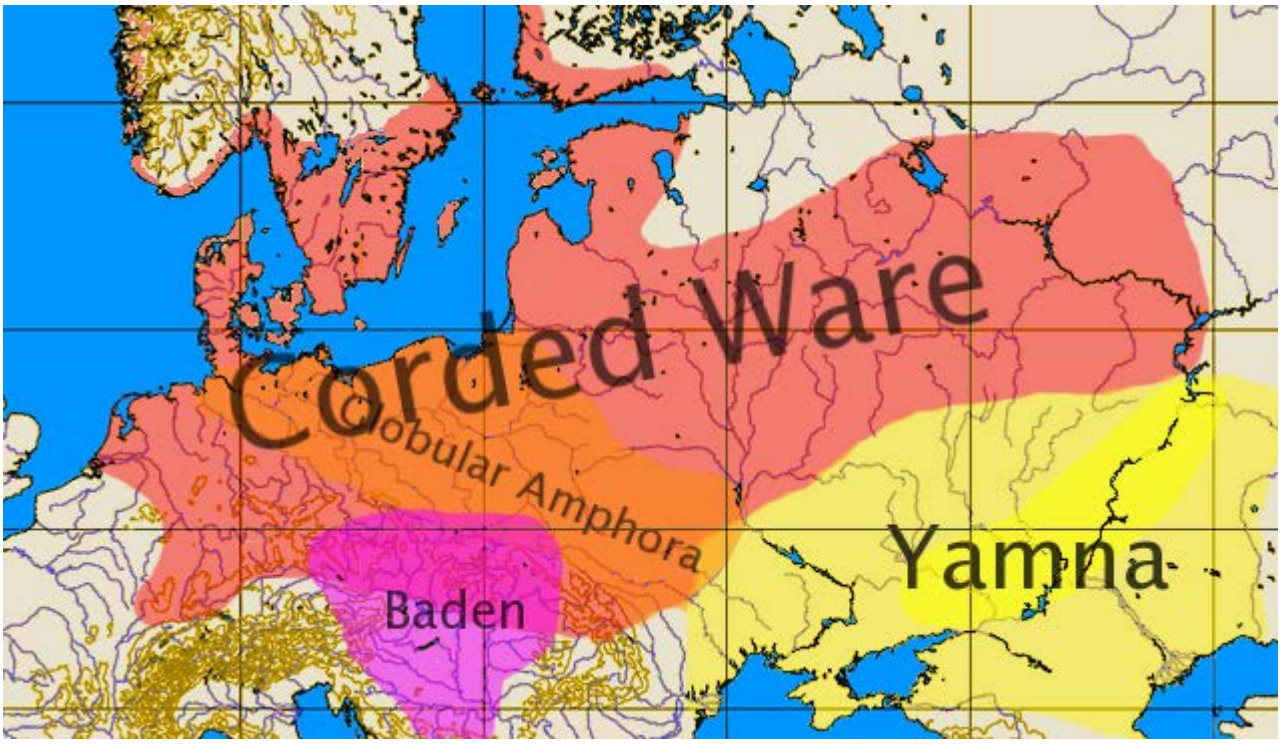


Image 1. Map of the main archaeological cultures of the Early Bronze Age in Central and Eastern Europe.

Initially these cultures dated back to the second half of the III–early II millennia BC (ibid), but nowadays the lower edge of their emergence shifted to the last centuries of the V millennium BC [**2, p 127**], although this shift can be explained by the calibration of radiocarbon dates, thus, it can have polemic character [**3, p 107-111**]. Initially the corded tribes were considered nomadic-pastoral, but today they are referred to as settled pastoralists and farmers [**1, p 35**], moreover, it is prerequisite by the forest area. «*Anthropologic composition of Corded Ware population is diverse. But the evident archeologic similarity indicates the common ancestor of these cultures. Different researchers sought this ancestor in Northern Black Sea Region (Gimbutas, Danilenko), in the Right-Bank Ukraine (Artemenko, Sveshnikov), in Polesia (Denisova, Kraynov) and in the interfluve of the Vistula and the Rhine (Fisher, Hoysler)*» [**ibid**]. Special attention is attached to the fact that over 20 ethnically related cultures have emerged within the distribution area of the Corded Ware tribes as a result of their inclusion in the local substrate [ibid]. So the problem of the genesis of the Corded Ware culture is complicated and a hypotheses concerning the Eastern impact on its formation is discussed.

Materials and Methods

The main research method of this paper is the interpretation of recently obtained genetic data which is compared with archaeological and, in lesser degree, linguistic data. The most used methodological approach is the use of the maps of ancient or modern spread of mtDNA haplogroups C and Z and Y-DNA Q, N, and R with the spreads of Corded Ware culture and its Eastern analogues. The serious methodological support is obtained by the use of the theory of A.A. Romanchuk of the migration path of Sino-Caucasian tribes and their origins in the Eastern part of Eurasia*. The proven areals of the ancient archaeological cultures and technologies compared with haplogroup areals are the main scientific materials for this work.

* Another eastern migration idea was independently proposed in http://pereformat.ru/2014/04/arbins/ in context of R1b Y-haplogroup migration

Discussion and Results

1. Corded Ware Substrate Problem

Substrate issue in the archeological cultures of Corded Ware and their possible descendants (Soviet monography of 1987 diplomatically points at the role of corded tribes as a basis of the Slavs, the Balts and the Germans ethnogenesis within the framework of the concept of autochtony of the major population [**1, p 37**]) deserve special attention. Linguists in cooperation with archeologists noted that the Balts and the Slavs historically have more similar features with pro-Germans, than with other Indo-European groups [**4, p 11**]. However, ethnogenesis of the ancient Germans (Jastorf culture [**5, 309-311**] is considered their habitat both today and in the XIX century [**4, p 19**]) appears to be complicated. Gradual transition of the preceding Neolithic cultures into the corded community [**1, p 35**] points at the special role of the so-called substrates. It is evident from the abundance of mito-haplogroups, inherent to this culture.

To 2015, different genotyped representatives of the Corded Ware Culture showed such diverse mtDNA haplogroups as H5a1, J1c1b, J1b1a1, K1a2a, K1b1a1, T2e, U4, U5b1c2 x [7], H6a1a, U2e1, U5a, U5a1, W6, X2, J1c, J2b1a, T2b и X2 (http://suyun.info/index.php?p=ancientdna).But the most of the Corded Ware cultures are characterized by the steady dominance of Y-DNA R1a1 haplogroup*.

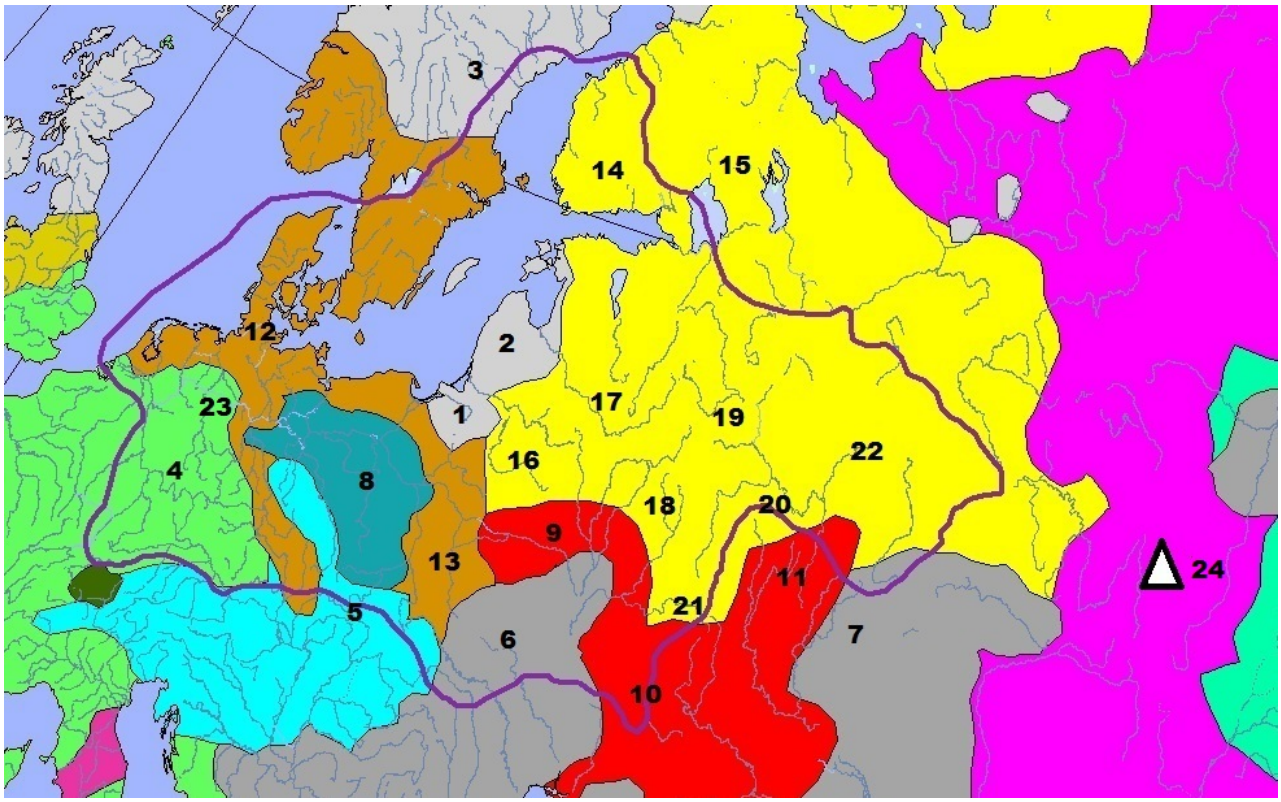


Image 2. The area of the Corded Ware culture and the absorption of the archaeological culture of the period around 3000 BC (violet line) according to 1, p 35-77 6, p 1231-1232.

1 – Zedmar culture
2 – Narva Culture
3 – mezolit of Scandinavia
4 – Michelsberg culture
5 – Lengyel culture
6 – Trypolie culture
7 – Khvalynsk culture
8 – Komornicze and Choiniki-Penkovo culture
9 – the late Dnieper-Donets culture
10 – Sredni Stog culture

* http://polishgenes.blogspot.ru/2015/06/bad-asses-of-bronze-age-analysis-of.html (see Supplement 1)

11 – Middle Don culture
12 – Funnelbeaker culture
13 – Globular Amphoras culture
14 – Sperrings culture
15 – Karelia culture
16– Neman culture
17 – Usvyaty culture
18 – Desna culture
19 – Balakhna culture
20 – Ryazan culture
21 – Ryboozerskaya culture
22 – Volga-Kama culture
23 – Walterninburg – Bernburg culture
24 – Sintashta complex

The same source poits out that «*R1a1 appears to be an Eastern Hunter-Gatherer (EHG) marker that in all likelihood failed to penetrate west of present-day Ukraine until the Late Neolithic, because it's missing in all the relevant samples before this period. So it probably first arrived in Central Europe with the Corded Ware people. We know that the Corded Ware people were foreign to Central Europe because their genome-wide genetic structure is starkly different from that of the Middle Neolithic farmers who lived there before them….* ***On the other hand, the Corded Ware sample, also from east-central Germany, is sitting at the other end of the plot, among groups from the Volga-Ural region***» The very important work [7] using wide range of paleogenetic data showed the crucial role of the Corded Ware in the forming of different indoeuropean groups, and especially those of Sintashta (Image 2).

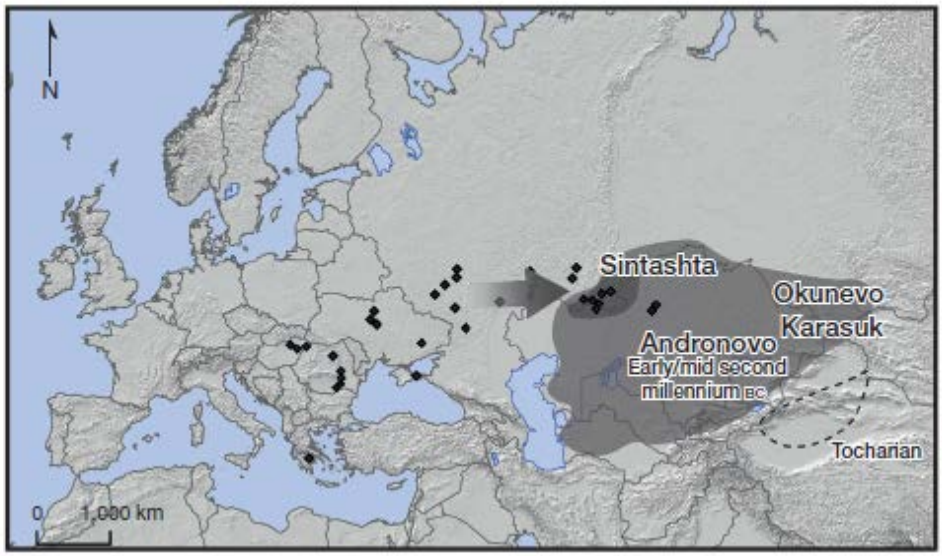


Image 2. The possible way of influence or migration between the Corded Ware and Sintashta [7].

The second important result is that Corded Ware cultures had at the beginning dthong impulses from Yamna (althoung, up to day almost all Yamna genotyped males were R1b1). The possible migration is outlined on Image 3.

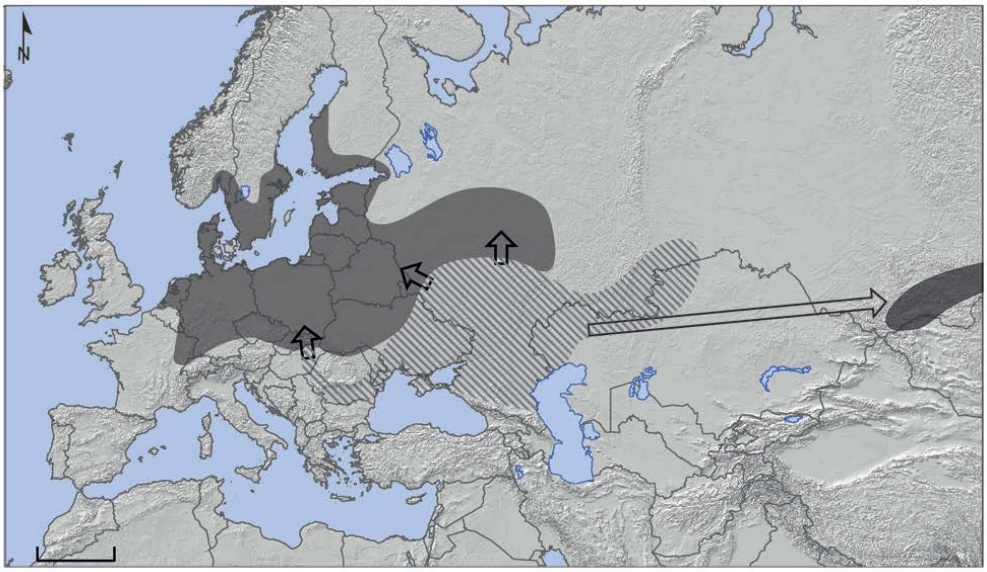
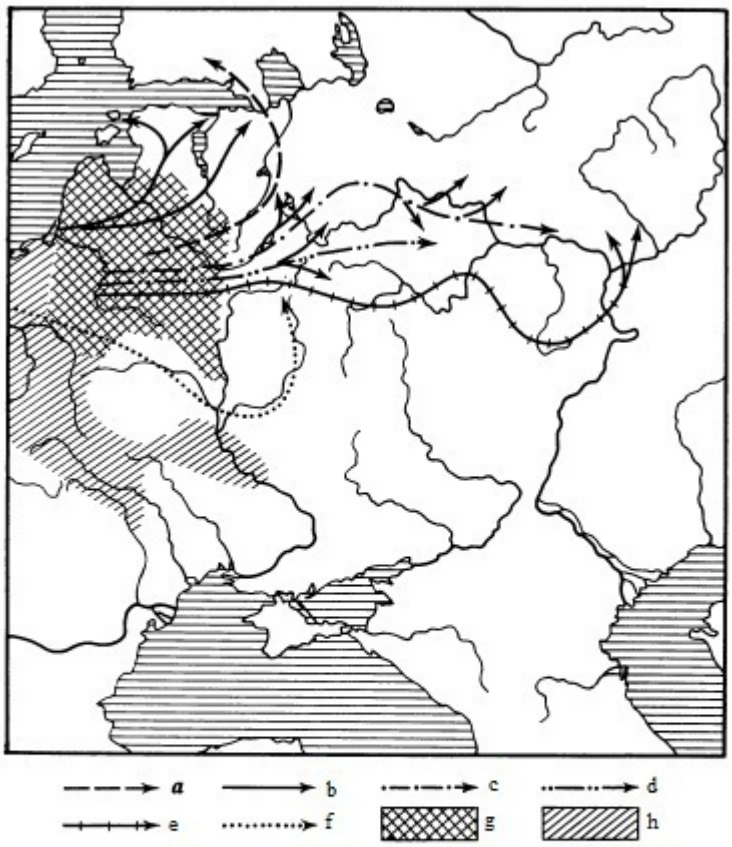


Image 3. The possible way of influence or migration between Yamna and Corded Ware [7].

Comparing this paleogenetic data with the localization of the initial center of the Corded Ware (or Battle Axe) culture, the Soviet monograph «The Bronze Age of the Forest Zone of the USSR**»** states:«The only initial territory possible is the one in the middle of the area of Battle Axe culture distribution, in other words, the territory between the Dnepr and the Vistula-Oder (Belarus, Ukraine behind the Middle Dnepr, the Carpathian Region, Poland, Lithuania, Kaliningrad Region and a part of Latvia), where all ancient components of these cultures are located. Their resettlement to the west and to the east – to Denmark and Cisurals area started from here» [**1, p 74**]. Also, we come across the migration of Yamna in the north-western direction (Image 4).



Map 11. The migration of Eastern branches of Corded Ware (Battle Axe) culture
a - Baltic group: b - Ilmen group: c - Upper Volga group: d - Volga-Klyazma Group: e-f - Middle Volga Group: g - proto Fatyanovo territory: h - Corded and Globular Amphora Carpathian and Baltic Group.

Image 4. Map of the tribal migration of the Corded Ware culture [**1, p. 72**].

The mentioned above habitat has, primarily, determinate relation to the territories, considered the time of Indo-European tribes' formation (firstly, the ancestors of Indo-Iranian peoples, including the Pit Grave culture) in the Neolithic epoch by L.S. Kleyn, expecting the population of the Funnel-beaker culture (or its part) to be the earliest Indo-Europeans [8, p 93, p 96]. Secondly, Sertey Neolithic complex is very interesting and significant for the neolitization of the vicinity of the east of the Middle Europe and it is located in the region, directly adjacent to the area, detected as the primary area of the further spread of the Corded Ware cultures, namely, the region of the north-west of the Smolensk Region (Dvina-Lovatsky interfluve). Here we can observe the sequence of the Neolithic cultures, although having different origin, but stratifying and interacting between VI and II millennia BC. These are the alien Early Neolithic Sertey culture [9, p 99-106], dated back to the Atlantic period 1-2, namely VI-V millennia BC [10, p 11-12], Dubokray type, connected with the cultures of the stroke-ornamented ware of the Middle Europe [11, p 108-110], dated back to V-IV millennia BC, Rudnyanskaya culture of the local Mesolithic basis [12, p 258], Zhizhitskaya culture, determined by A.M.Miklyaev, and North-Belarus one (II millennium BC) were deeply influenced by the Corded Ware culture [13, p 23]. We should note that the transition period from the middle to the late Neolithic Age (namely to the North-Belarus culture) in Sertey is occupied by the so-called Usvyatskaya culture, having much in common with the Funnel-beaker cultures, or being their eastern edge [14, p 9]. The research (a tooth was found in the grave) detected that the population of Sertey culture could have the male haplogroup R1a1[15, p 294]. Is it the target source of subclade R1a1, spread in the habitat of the Corded Ware tribes? Of course, the multiple migrations in the region, especially the possible migration of the carriers of the same

haplogroup from the south-east, from the region of ancient Yamnaya-like tribes in the initial period of their history should be also considered. It is closely related to the above mentioned tooth, the carrier of the male haplogroup R1a1, referred to the layer of Sertey VIII (ibid), which is much younger than the epoch of the Sertey culture (Sertey XIV-X) and related to the Usvyatskaya culture, determined by Mazurkevich, connected with the stroke-ornamented ware culture from the Middle Europe, neither than the northern Caspian Sea Region and the Middle Asia [14, p 9-10].

This article attempts to study the archeological issue, concerning the origin of the Corded ornament of the ware (all the abovementioned cultures were defined in accordance with it) in Neolitic/Eneolitic cultures.

The Sperrings culture is perhaps one of the most ancient candidate for the role of such ornament source in the Western Europe Region [16, p 79], which existed in the second half of V–IV millennia BC and partially the habitat of the pit-comb ware [16, p 123]. The corded ornament was found in Sperrings. If we consider the theory of the origin of the corded ornament in the pit-comb ware technique more thoroughly, we can suppose some (though, very indirect) roots of the ornament in the Dnieper-Donetsk culture, which is regarded as the source of the pit-comb technique (which is, appearingly, was not accompanied with migrations of any kind).

The Sperrings culture has local background [17, p 212], which, of course, includes the alien influence. The cultural body of pit-comb ware spread along the north of the Western Europe from the Dnieper-Donetsk cultural community, though the ware of the latter does not contain the corded ornament. Thus, precisely the Karelian-Finnish late Neolithic Sperrings culture can be considered one of the main candidates for the role of the technological source of the Corded Ware in the Western Europe. The other candidate is the Eneolithic ware of the Sredny Stog culture (primarily – the location of Lipetsk Lake, for which the corded ornaments, besides the rudiments of pit-comb technique, vaguely resembling the classic variant, were typical). This type of ornament recedes to the past of the region – to the early Neolithic Age of the Upper Don [18]. Thus, the tradition of ware decoration with the Corded ornament existed in steppes and forest steppes of the South of Russia in the VI millennium BC and could spread to the North within the frameworks of either pit-comb ware, or directly to the region of the Battle Axe/Corded Ware cultures formation, namely, to Belarus-Poland-Western Russia. The remained theories take us outside of the region, primarily, to the Southern Trans-Urals.

We can get more accurate picture if we analyze the second, Eastern center of the corded ware development.

2. **Eastern Asian Center of Corded Ware**

Links with the East and the Eastern Eurasian influence on the Corded Ware culture (both proved and hypothetic) touch upon one of the most interesting problems of the Siberian Neolithic, namely the problem of Corded Ware ornament of Bel'kachinskaya culture in Yakutia [**17, p 298**]. According to Everstov: «*Bel'kachinskaya culture people made their pottery by knocking it out. While forming the vessel, they used the wooden stick with the twisted cord tied around the working tip. Sharp prints of the latter stamped at the surface of a vessel. The scientific literature calls the pottery, made by the cord tied around the wooden stick the corded ware. The remains of the corded ware are found within Yakutia, as well, as at the Far East and even in North America. The researchers therefore suggest that Bel'kachinskaya culture people could be the ancestors of some Indian tribes of North America*» [**19, p 40-64; 20, p 66**]. One of candidates for these tribes could be Na-Dene, as they are considered to be one of the latest wave of the New World migration form Asia.

Image 5. The map of the Bel'kachinskaya culture habitat (pink)

Let us clarify the dynamics of the overview of the Yakut Neolithic. Bel'kachinskaya Neolithic culture dates back to the IV-III millennia BC. (3200-2100 BC) [**20, p 49**]. It occupied almost the whole territory of the modern Yakutia. Syalakhskaya culture is considered its predecessor and the closest in its characteristic features [**17, p 298**)] in the same Yakutia territory (formed in the Middle Lena basin in V-IV millennia BC as a result of migration of the tribes from Transbaikal, which assimilated the preceding local Sumnaginskaya culture; more correct it is dated back to 4500-3200 BC [**20, p 49]**). The newer datae of the discoveries, belonging to Syalakhskaya, Bel'kachinskaya and Ymyyakhtanskaya cultures have significantly complicated the clear scheme mentioned above. Now these cultures seem to co-exist during the long period, rather than interchange (which is, taking into account the vast expanse of Yakutia is not surprising). Consequently, the dates have also changed: «Syalakhskaya culture (early Neolithic) – 4870 ± 170–3490 ± 150 BC (duration about 1380 years); Bel'kachinskaya (Middle Neolithic) – 4100 ± 300–2160 ± 150 BC (about 1940 years); Ymyyakhtakhskaya (Late Neolithic) – 2900 ± 450–1025 ± 235 BC (about 1880 years)» [**21, p 27, p 34**].

According to Yu.A. Mochanov «*the carriers of Syalakhskaya were the ancestors of Nganasans, residing Taimyr*» [**22, p 174**]. It seems possible of the mutual influence of Syalakskaya and Isakovskaya cultures [**22, p 164**]; Isakovskaya culture of the Developed Neolithic, showing the genetic similarity with the preceding Kitoy Early Neolithic culture [**23, p 1418–1425**], dating back to the late V– IV millennia BC. Nganasans as the descendants of the Neolithic hunters on the reindeer are really connected in their origin with the basins of the Middle and Lower Lena, where from they penetrated into Taimyr 4000 BC [**24, p 242**]. But the origin of Nganasans is mixed, as different tribal groups integrated into their contingent until very recently (Pyasinskaya Samoyad', Kuraks, Tidiris, Tavgs) (**ibid**). It isn't proved that Nganasansky language, belonging to the Samodiyskaya group of the Uralic family was definitely the language of Syalakhskaya culture of the Yakut Neolithic. With regard to paleogenetics no links of Bel'kachints-Syalakhs are found now. Studying mitochondrial DNA of the modern Yukaghirs, which inevitably had to perceive the gene pool of the preceding Syalakhskaya and Bel'kachinskaya cultures, N.V. Volodko came to the conclusion that «*1.Mitochondrial gene pool of the Yukaghirs consists of East-Eurasian haplogroups – A, C, D, Z, and G. Haplogroups C and D are the most frequent and diverse. The new haplogroup D2*, undescribed before, was found. It witnesses the genetic imprint, left by the migrations of early Eurasians in the interfluve of the Lower Kolyma and the Indifirka. 2. The ancestor haplotype of he haplogroup C2a was identified in the Yukaghirs of the Lower Kolyma. Its age (8150 thousand years) coincides with the beginning of the re-colonization process of the Siberian Arctic and Subarctic Regions … Age-related estimation of the haplogroups C and D, dominating in the mitochondrial landscape of Siberia coincides with the existence of the Late Paleolithic Selemdzhinskaya culture of fishermen and hunter gatherers, formed in the basin of the middle reaches of the Amur approximately 25 thousand years ago*» [**25, p 15-16**].

Migrations of Samoyedic peoples to the west add a personalized touch to the hypotheses, concerning Sami origin (despite their linguistic affinity to the Finns, they seem to have a significant substrate). «*According to one hypothesis, Sami have not initially belonged to Finno-Ugric peoples. In ancient times this Arctic nation came in contact with Sami tribes, further it interacted with Baltic-Finnish tribes and adopted their language (well-known Russian Finno-Ugric researcher D. Bubrikh, while considering this issue, highlighted that Proto-Sami tribes had contacts with Samodiets, but did not have genetic relationship*» [**27, p 43**]. It is not only the matter of paleogenetics, but paleolinguistics, as well. We probably face the problem of the so-called pre-Finno-Ugric substrate, which was searched for in different language families– for example, Tungusic subfamily [**28, p 20-24**], though the hypothesis, concerning the fact that Y-DNA subclade N1b (including the ones of Tungusic peoples, where it is present) could be the marker of pre-Finno-Ugric substrate, would be too courageous.

Comparative genetic analysis of the ancient cultures and the modern ethnic groups of Siberia does not help to identify them completely. Male Y-DNA haplogroup N is widespread in the North of Eurasia and concentrates in various ethnic groups: Balts, Finns, Yakuts [**29 – 31**]. Certain subclades of this haplogroup are the most frequent among the peoples of the Extreme North of Russia: nomadic Nganasans belong to the Y-haplogroup N1b (so-called Samoyedic) by 92 % and to the Y-haplogroup C by 5 %. This is the highest rate of the haplogroup N1b among all the peoples [**30; 32**]. It is supposed that N1b emerged in 2500-8000 BC within Sayans or nearby. «*But we should also admit that the resemblance between Samoyedic and Turkic-speaking N1b-carriers is rather remote and goes back to the Neolithic Age (3000 – 3500 years ago). Turkic languages are rather young, if compared to Indo-European, Finno-Ugric and Samoyedic ones. The possible homeland of the Turkic languages coincides with the homeland of the haplogroup N1b could be Mongolia. Thus, Turkic-speaking N1b carriers should be considered as the initial speakers of the pre-Turkic language, genetically close to pre-Samoyedic peoples, who have preserved the common ethnonyms with Samoyedic peoples from the ancient times, rather than Turkified Samoyedic peoples*» [**33, p 405**]. If the authors of the quoted article are right in the chronology of the divergence of Samoyedic and Turkic-speaking N1b carriers, we can suppose that this split (the second half of the II millennium BC) is younger than Bel'kachinskaya and Syalakhskaya cultures and the theory by Mochanov that Bel'kachinskaya peoples are predecessors of Nganasans doesn't concern with the genetic affinity.

A.A. Romanchuk has touched upon another grand challenge of the paleogenetics of Yakut cultures (including Dene-speaking) as he noticed that all Indian tribes had migrated before Y-DNA N1 carriers came to the North-East Asia. Dene-speaking groups within the American continent do not belong to the N1b Y-subclade dominant group or have any presence of it. Y-chromosome Q (respectively 78.1%, 70.4%, 92.3% and 64%) [34; 35] dominate in Apaches, Athapascans, Navahos and Tinklits, R1 (62.5%) [36] in Chepivyans. Y-haplogroup N on its own occurs only in Eskimos, but their emergence in the New World dates back to latter time. If the tribes of the mentioned archeological cultures of Yakutia (who according to Everstov penetrated to the North America) were Dene-speaking and brought some wave of Na-Dene languages to the New World, they did not bear any share of N1b Y-DNA subclade. That means that their migration to America occurred earlier than this N1b subclade expanded across Yakutia, or that the subclade completely disappeared in the conditions of America (what is less probable). It is interesting that the explanation of the existence of R1 subclade in North America is considered unclear: the theory has it that the white colonists contributed R1 to North America [37], but the hypothesis on the links between American R1 carriers and Mal'ta-Buret' culture seems more likely to be the truth. This culture features subclade [38], suggesting that subclade R1 together with Q, has been a characteristic for the Amerindians, which is the major part of American Indians with no micro family-scale language relatives in Eurasia.

Samoyedic peoples appeared in the Arctic Circle rather late, after the beginning of our era, in the V century BC their ancestors inhabited the Middle Ob (Kulay culture) [24, p 250], and consequently had no concern with neither Taimyr, nor Yakutia. Thus, we can speak of the language change and assimilation of the resettles from the Lena to Taimyr. We consider the opinion of Mochanov, concerning Bel'kachinskaya Dene-speaking (or other connection with the New World) and possibly of the Syalakhskaya culture, preceding it, much more promising.

Nowadays peoples of the Na-Dene language family inhabit the greater part of Alaska, north-west of Canada and occur in a number of American states at the Mexican border. During the XX century different linguists (E.Sepir [39], S.Starostin, E.Vayda [40, p 177-178], J.Grinberg, M.Rulen and others) independently of each other came to the conclusion that Na-Dene is close and related with Sino-Tibetan and Yeniseian languages (according to Starostin and Grinberg, the two latter families are related to each other). The general protolanguage for the Yeniseian and Na-De languages exited in the epoch of the late Mesolithic Age (ibid.). The fact that nowadays (excluding Eskimo-Aleut family) no language taxon of a family scale lives both in the Siberia and in the North America is one of the most interesting issues in the matter of Siberia-American ethno-linguistic migrations, which demands explanation. For example, if we find peoples in Siberia, relatives of the American Indians in terms of language, or, vise versa, Nganasans (if they constituted the essence of Syalakhskaya culture and later migrated to America) occurred in Canada or Alaska, we could easily trace the chronology and geography of such migrations. As it is not observed, we can make the conclusion that the migration of the Siberian peoples (except Eskimos) to America ceased no later than 5000 years ago and the remnants of Amerinds and Na-Dene in Siberia assimilated long ago.

The problem of the whole Dene-Caucasian macro-family migration and the matter of these migrations chronology are concerned with the problem of Dene-Yeniseian language community. There's a traditional point of view, according to it the ancestors of the Chinese men, Tibetans and Na-Dene migrated from the Middle East and South Asia in the early Mesolithic Age. But there is a new theory by A.A. Romanchuk, according to it the direction was opposite and belonged to the earlier epoch -Upper Paleolithic [41]. But, no matter which of the theories is proper, Yeniseian languages (as a part of Dene-Caucasian unity) inevitably occur in the central zone of the hypothetic migrations, specifically in the Middle Siberia and probably had contacts with Na-Dene tribes in this region. These contacts dated back to the Mesolithic Age (taking into account the chronological anachronism of the Siberian Mesolithic Age). Perhaps, Western Siberia, South Asia and Urals (at least partially) were the zone of Yeniseian tribes in the Mesolithic and Early Neolithic Ages and Na-Dene tribes neighbored to the east, inhabiting the greater part of the Yenisei and the Lena basins. And the vicinity of Baikal is considered the traditional zone of the Altai tribes' formation.

As the formation of the Syalakhskaya culture, preceding the Bel'kachinskaya one is connected with Trans-Baikal, we should turn in our searches of the initial migration zones of hypothetic Na-Dene (or another Indian) tribes related to Bel'kachinskaya to archeological periodization of Trans-Baikal history of the V millennium BC. The Neolithic Age of Trans-Baikal significantly deviates from the Neolithic Age of that of the Baikal Region [17, p 306], and the first Neolithic period (the so-called Mukhinsky stage) started not until IV millennium BC. Thus, searching for the homeland of the Syalakhskaya Neolithic Age we can speak of the late Mesolithic Age of the region, rather than of the Neolithic Age. Although, the first traces of Neolithization of the south of the Western Trans-Baikal, in the region of Studenovskaya Upper Paleolithic and Mesolithic culture date back to the middle of the V millennium BC. It may happen under the influence of the surrounding, more ancient Neolithic cultures (primarily, the Amur ones) [17, p 307]. It is striking that the ceramics of two types: "*textile and knock-out corded ware, which is stated in a number of Early Neolithic sites of Baikal and Angara Region*" are typical for the Mukhinsky stage (ibid). Within Vitim Region the corded ware has remained in the Late Neolithic period (i.e. III-II millennium BC): the utensils were handled by the knocking-out with the wooden stick, the same way as in the Bel'kachinskaya culture [17, p 309]. Thus, the corded ware has already existed within the territory of Trans-Baikal (possibly, Kitoy, because Kitoy people have come to the Baikal Region from Trans-Baikal [17, p 271]) and Tungusic (17, p 310) Neolithic tribes not until V millennium BC (uncalibrated). The corded ware spreads from Transbaikal Region to Yakutia and Baikal Region. The origin of the creators of Trans-Baikal Neolithic should be searched for in Mesolithic culture of a kind of the Upper Lena Mountain [10, p 197]. Perhaps, they were the Early Mesolithic Na-Dene ancestors, although Kitoy people are more commonly referred to the ancestors of Altainian people (in any case, we can state very close contacts of Na-Dene and Altainian people in Mesolithic and Neolithic Ages).

Systematic study of Bel'kachinskaya culture has been conducted since 1960s. Ya.A. Molchanov is considered the acknowledged expert in this field of study. He competently examines Bel'kachinskaya culture and its setting in his rather old monograph [21]. The corded ware in the Neolithic Age is typical for Japan, Sakhalin, Trans-Baikal, Angara, Amur, Ussuri, the Kurile

Islands, but only Japanese, Sakhalin and Transbaikal types are similar to Bel'kachinsky one [22, p 180]. The corded ware in Japanese Jomon emerged in the III millennium BC [22, p 181]. Thus, the phenomenon we are interested in, namely the corded ware is typical for a rather vast range of Neolithic cultures in the area of approximately ancient center of primeval 'Boreal' ceramics (in modern estimations– XI-X millennia BC)

Minimum two centers of the so-called 'pseudo-string' ceramics existed in Eurasia. It can be considered as the preceding form of the classic string one. It is the Altai location Boynikha, dated back to VI-V millennia BC and the one, synchronous to the Neolithic settlement Tytkesten'-VI [43, p 114-116 and the whole South-Ural and North-Kazakhstan Eneolithic community of comb ceramics, singled out by V.S. Mosin, including Surtadinskaya, Kysykul'skaya, Tersekskaya, Botayskaya, Ayatskaya cultures. They (Tersekskaya and Ayatskaya) are characterized by pseudo-string ceramic ornaments [43, p 183, p 227, p 237, p 283]. Referring to the origin of the pseudo-corded ware in the South-Trans-Ural Region, the authors of the monograph wrote: '*The Chronology of Eneolithic and the Early Bronze Age in the Urals Region*' *state: «At the turn of Neolithic and Eneolithic Ages, in the second half of the V millennium BC, retreating-stroke-ornamented component of Poludensky complexes has evolved in pseudo-string one, comb has evolved in comb Eneolithic one with simple and geometric ornaments*» [44, p 41-53]. We can also make an assumption, concerning the influence of the Far East center of the Early Neolithic within the Baikal Region, but, referring to the 'pseudo-string' ceramics within the Altai Region, remains the possible variant of western influence (from Trans-Urals), neither than eastern.

Mochanov expands Bel'kachinskaya culture to Chukotka and the Sea of Okhotsk [22, p 167, map]. Bel'kachinskaya culture affected the region of Anadyr basin (Ust'-Belaya)[22, p 182], but its influence goes beyond. In an old dispute of Mochanov and Diky, concerning the major source of North-American cultures and, consequently, the initial position of migrants to the New World via Beringiya or across the Bering Strait (although, we think that the dispute is pointless at least because, despite Eskimos, America is the homeland of the Indians of the four large linguistic taxons; that's why America was settled by the representatives of minimum four Eurasian groups), Mochanov defended the 'Yakut homeland'. The corded ware of Bel'kachinskaya culture has analogs in the culture of Woodland (east of the USA), but this influence is insignificant (ibid). The Bel'kachinskaya culture is much similar to the complex of the Firth River to the north-west of Canada on the banks of the Firth River – 30 km from the Beaufort Sea (III-II millennia BC) [22, p 183]. The Natwacruac cultural complex in the northern Alaska, dated back to IV-II millennia BC [22, p 178] is also similar to the Bel'kachinskaya culture. The modern American studies refers the first entries of paleo-Indians into the New World to the period of XX-X millennia BC (in other words, to the Upper Paleolithic) and even earlier. But in case of the Bel'kachinskaya culture and its corded ware, we deal with some kind of migration (ethnic or technologic), dated back to the latter time (not until late IV millennium BC). Such migrations must have occurred repeatedly (not only from the west to the east, but theoretically, in the opposite direction, from America to the Siberia). But, appearingly, this migration (excluding Eskimos), which occurred in late IV millenium BC, was the last significant Indian migration. No migrations of such scale are observed in the following five millennia (from our point of view, it explains the lack of Indian languages in the modern Siberia and the lack of Altai, Urals and the so-called paleo-Asian languages in North America, - 5000-year period was enough and the conection was lost

The problem of comparison of the above-mentioned American archeological cultures in the monograph by Mochanov with the modern ethnic groups of the American Indians directs us to the modern ethnic map of North America. Peoples of Na-Dene language family inhabit Alaska and the greater part of north-western Canada, in other words, the same territories, where Mochanov finds analogues to the Bel'kachinskaya ceramics (geographical position of Dene-speaking tribes points at the fact that they seem to be the last migration wave of the Indian population from Siberia. This is another argument in favor of the Dene-speaking of Bel'kachinskaya culture). The proximity of the Firth River complex to the Arctic Ocean coast suggests that Na-Dene people inhabited this region prior to the Eskimos.

Another language group of North American Indians attempted to compare with Dene-Caucasian people. After Nostratic and a number of different comparative theories have been introduced for the scientific use (and the emergence of larger taxons if compared to the traditional language family), the modern Indian peoples of America are divided into four large groups: first of

all, Amerinds, who have no relatives of macro-family scale outside America and are macro-family on their own, secondly, Hokan-Siouans, thirdly, Penutians, and, at last, Na-Dene, a part of large Dene-Caucasian macro-family. But reality is always more complicated. The whole macro-families are singled out among the families of Amerind macro-family (called 'philia' and the linguistic view resembles the 'division' of a large Altai family into the few smaller ones), including the ones, similar to the Eurasian macro-families. First of all, it is the so-called Mosan language macro-family, offered by E. Sepir in 1929 in the article, written for the British Encyclopedia. According to Sepir, Salish, Wakash and Chimakuan languages of the north-western coast of the Pacific Ocean are included into this macro-family (45). Although most linguists haven't supported the theory, it found a response in paleogenetics: «Similarly, a fourth non-local component found in most parts of Europe is from the Salishan region that includes indigenous populations of the Pacific Northwest of North America. Low levels of this component are found throughout Europe, with the largest percentages in the Urals (6.5%), Celtic (5.5%), Russian (4.5%), and Finnic (4.3%) sub-regions. These Salishan percentages do not necessarily suggest any direct links with Native Americans; instead, these might express genetic traces of early links between archaic European populations and the early Eurasian ancestors of indigenous Siberians and Native Americans (possibly dating to the Mesolithic period). Both Altaian and Salishan genetic components in Europe might reflect genetic traces of the Eurasian hunting-fishing cultures that were absorbed and pushed outwards by expanding Indo-European populations since the Neolithic period» (**46**). Thus, Indian, Salishan component is present in Europeans. It is especially strong in Slavic and Celtic environment, where subclade R1a and R1bв are most frequent (Celts have peak values of R1b), as well as in the Urals and in Finland. V. Shevoroshkin has compared Mosan languages (namely, Salishan language) and East-Caucasian ones (Naho-Dagestan language family) and determined the approximate time of their separation – 4000-5000 years ago (**47, p 88**). But, appearingly, such migration (if it really occurred) referred to the category of micro-migrations and should have remained almost inconspicuous in Siberia.

The attempts to compare the modern linguistic groups with the archeological cultures of the recent past were always hard, but the Bel'kachinskaya culture, followed by the Ymyyakhtakhskaya one (2100-1300 years BC – [**20, p 50]**; 2900-1000 years BC – [**21, p 27, p 34**]), within Yakutia and the adjoining areas is a mess: «*As of the ethnic background of the Ymyyakhtakhskaya culture carriers, it is still unclear. The existing working-level opinions are absolutely different. According to A.Yu. Mochanov, Ymyyakhtakhskaya people are the ancestors of the Chukchi and the Koryaks. This theory was later supported by I.V. Konstantinov, S.A. Fedoseyeva and V.I. Ertyukov. N.N. Dikov associates Ymyyakhtakhskie memorials with the ancestors of the Chukchi and R.S. Vasilevsky – with the ancient Koryaks culture. A.P. Okladnikov, archeologists L.P. Khlobystin, M.A. Kiryak, Everestov, ethnographers I.S. Gurvich, Yu.B. Simchenko and anthropologist M.G. Levin (1958) consider that the carriers of the Ymyyakhtakhskaya culture are the ancestors of the Yukaghirs. According to S.A. Arutyunov, the carriers of the Ymyyakhtakhskaya culture constituted the polyethnic historical and cultural integrity, including the Chukchi, the Koryaks, the Eskimos, as well as the Nganasans and the Yukaghirs. A.N. Alekseyev shares this opinion*» (http://m.sakha.gov.ru/node/16435). The reason for this disagreement is the territorial expansion of the Ymyyakhtakhskaya tribes, settling on the territories from Chukotka to the Lower Yenisei and the Middle Amur by the middle of the II millennium BC. But it doesn't mean that the population of the Ymyyakhtakhskaya culture is Chukotko-Koryak or Nganasan. The prevailing element in this culture, were, probably, Yukaghirs, related to the Urals language family, who somehow showed up in the Baikal Region in late III millennium BC (this issue demands further research), wherefrom they came to the Lena basin [**24, p 416**]. Yukaghirs prevailed in the Eastern Siberia to the beginning of AD, followed by the Evenks (ibid), who came from the Baikal and Trans-Baikal Regions, as well. This is the amazing kaleidoscope of the ethnic map of the Eastern Siberia. The most possible way is thatDene-speaking Bel'kachinskaya people were assimilated by the alien Yukaghirs, having remained within Taimyr (Maymechinskaya culture) for some time. It should be emphasized that Na-Dene is the only community, whose the folklore has some undoubtful analogies in South Siberia and Central Asia and no analogies in the South America [**48**].

3. Possible Connection Between two Centers of the Corded Ware

Of course, the above mentioned facts of the corded ornament emergence and development in the ware of a number of Neolithic, Eneolithic cultures of the east and west of Eurasia demand plausible explanation. Could we in the similar vein suppose that the corded ware within Eurasia in the Neolithic Age could have two (at the minimum) advent centers or should we search for the links between the Far East Corded Ware center and the Western cultures (for instance, Sperrings), keeping in mind the so-called 'pseudo-corded' ware of the Southern Trans-Urals?

If the corded ware of the Far East (and the ware of this region in general) dated back to the earlier period, than the Eastern European one, we have the reasons to consider the possibility of one or another (at least specifically-technologic) migrations and vice versa. To solve this issue we need to compare the emergence of the corded (and pseudo-corded) ware in different regions of Eurasia more or less clearly.

'Pseudo-corded' ware of Altai – VI millennium BC
Sperrings culture – late V-IV millennium BC
Corded ware of the Eastern Europe – III millennium BC
Corded ware of the Baikal Region – V millennium BC
Corded ware of Trans-Baikal – VII millennium BC (minimally, because Trans-Baikal in this regard is older than Gromatukhinskaya culture)
Corded ware of the Middle Amur (Gromatukhinskaya culture) – VI millennium BC (minimally, because today Gromatukhinskaya culture dates back to the XIV-V millennia BC)
Corded ware of Yakutia (Syalakhskaya and Bel'kachinskaya cultures) – V-III millennia BC
Corded ware of the Eneolithic Dnepr Region – IV millennium BC
'Pseudo-corded' ware of the Southern Trans-Urals – second half of the IV millennium BC
Corded ware of Jomon culture – III millennium BC (though, ancientifying, similar to the one of the Gromatukhinskaya culture is also possible)

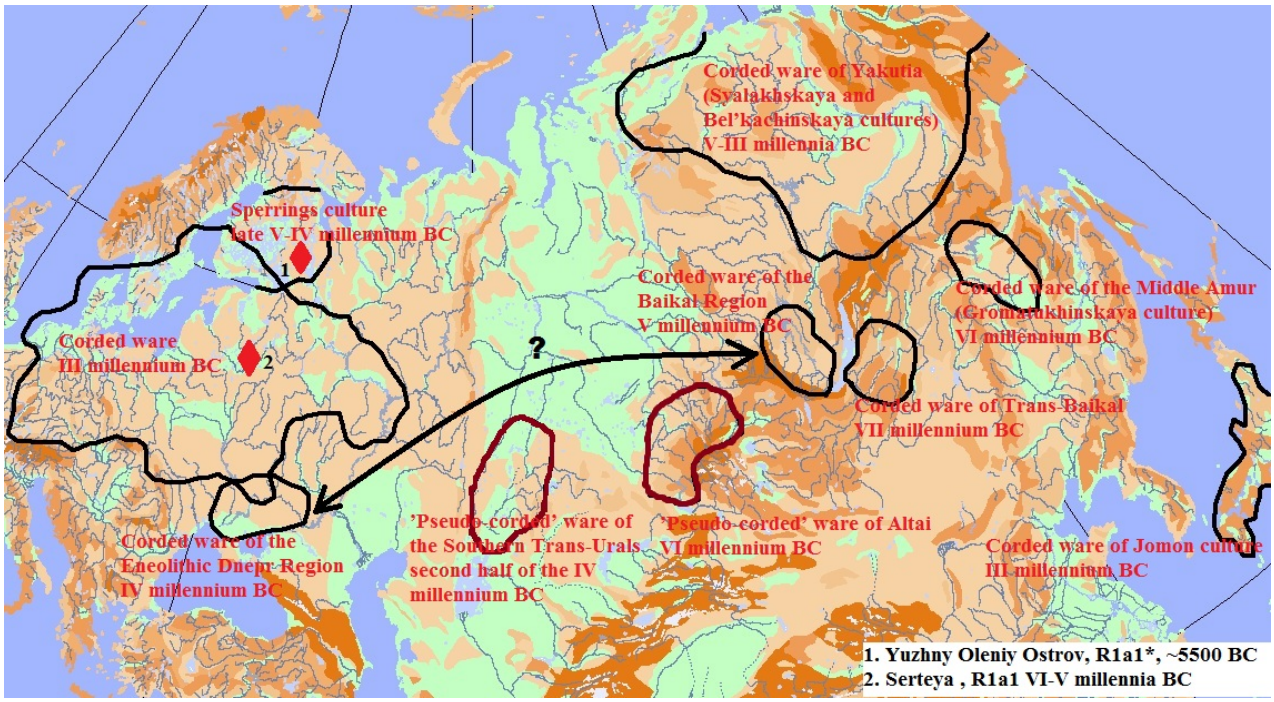


Image 6. Corded Ware cultures in the Neolithic and Chalcolithic Eurasia. The arrows indicate the possible migration. The distribution area of the real corded ware is colored in black, the 'pseudo-corded' one – in brown.

On the basis of the map, two explanations are possible:

1) Two centers of the corded ware – East-European, including the Sperrings culture and Middle Don and the Neolithic center in Trans-Baikal, which did not contact with each other, being

separated by great distances and the lack of the transition forms (if we don't consider 'pseudo-corded ware as one) existed within Eurasia.

2) The Far East Neolithic center in general and the corded ware in particular is older than the West Eurasian one and it affected the latter. These influences can be of two types: a) highly technological migration, which didn't affect the paleogenetics, paleoanthropology, paleolinguistics and the general archeology, б) notable migration, having affected paleogenetic, paleoanthropologic, paleolinguistic maps of Neolithic Eurasia.

Our goal was to find these traces and their possible paleogenetic trace.

First of all, we have detected the interesting anthropologic correspondence between the population of Dnepro-Donetsk cultural community (V-IV millennia BC) and the Altai Neolithic population [**50, p 19**]. If compared to other Neolithic anthropologic types, they are rather close to each other.

Secondly, the traces of the possible migrations from the Eastern part of Eurasia can be seen on the example of Karelia-Finland (and the Sperrings culture*). The Ice Age, in general opinion, ended about 10000 BC (plus-or-minus 500 years, but Finland completely cleared from ice only in the VIII millennium BC [**10, p 27**], and was occupied by the Mesolithic tribes [**51**] at that time. There are two main theories, concerning the settling: firstly, The Mesolithic population came from the east (from Ural or even from Trans-Ural) and, secondly, the region was settled from the south and from the west [**10, p 27**]. The distinguishing feature of Finland settlement is the presence of the large periglacial Ancylus lake [**52**], which could be either the hindrance or the way for the settlement (in terms of navigation development in the coastal Mesolithic tribes). Archeologists note the small number of evidence in favor of both the first and the second theory [**10, p 27**] and the popular theory, according to which the so-called 'pre-Finnish substrate' was followed by the modern Finns obscure the general understanding. It is commonly thought that only the cultures of the textile ware (1500-500 BC) were main predessesors the real Finns of the Volga and the Baltic regions [**53**]. Thus, all the preceding segment of the people, namely Volosovo-Garino-Borsky Eneolithic (III-II millennia BC), pit-comb ware (IV-III millennia BC), forest cultures with comb ware traditions (V millennium BC) [**54, p 73, 77, 83; 55**] and the earlier Mesolithic tribes (in fact from the moment of Finland and Karelia settlement in the VIII millennium BC) are exactly this 'pre-Finnish substrate' (more likely several substrates)

Paleogenetics has suggested another facts in favor of the eastern wave of the settlers. The Yuzhniy Oleniy Ostrov burial ground in Karelia proposed the researchers more riddles, than answers and sprang many surprises. For instance, three people from the Oleniy Yuzhniy Oleniy Ostrov, who lived 7500 years ago (UZOO-7, UZOO-8 and UZOO-74), were the carriers of mitochondrial haplogroup C1, very rare in Europe. Haplotype of the three inhabitants of the Yuzhniy Oleniy Ostrov refers to the special new subclade C1f. No concordances with this subclade were found in the current database of the ancient and modern mitochondrial genomes [**56, 65**]. Female mitochondrial haplogroup C is general in the North-Eastern Asia (including Siberia) and is one of the 5 mitochondrial haplogroups, found in the aborigines of America, along with A, B, D and X. It suggests that the Mesolithic migrations from Siberia to the north and north-east of Europe were real. This genetic migration can be compared with the migration of the carriers of the male subclade R1, typical for both the Eastern Europe and the center of Asia (**57**), and according to the primary data (hereunder), R1 can be found in North America. The spread of this subclade from the center of Asia is the widely-accepted opinion. Y haplogroup R1a1* - M198- along with mito-haplogroup C1f (one person) were found in the burial ground of the Yuzhniy Oleniy Ostrov. This is the first argument in favor of their collective spread from Asia to the North of Europe. The articles by V.I. Khartanovich and A.A. Romanchuk [**58**, **59**] contain arguments in favor of the north anthropologic type and in favor of the belonging of the population of the Yuzhniy Oleniy Ostrov to the North-European relict odontologic type.

* In **[68, p. 24]** is given a conjecture with some arguments that the people of Yuzhniy Oleni Ostrov could know ceramics and some of them could be essentially the early representatives of Sperrings culture. The dating of Yuzhniy Oleni Ostrov corresponds to the beginning of the ceramic neolite in North Eastern Europe.

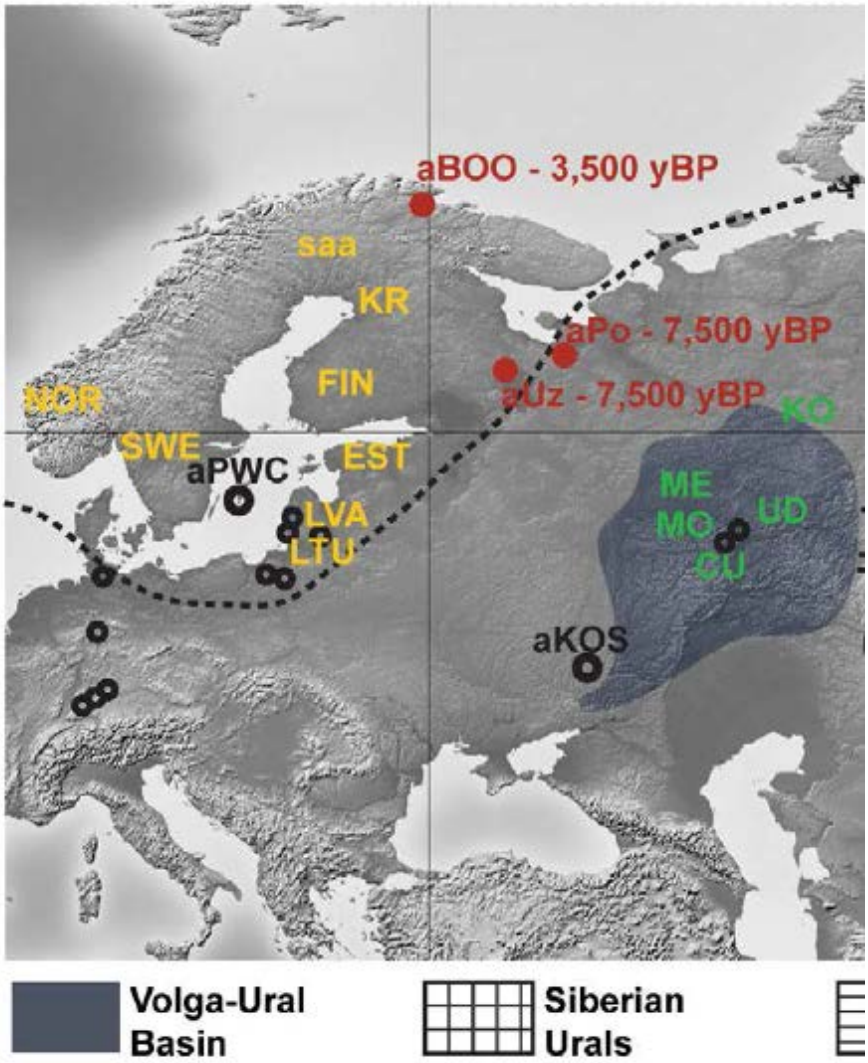


Image 6. Map of Eurasia showing the approximate location of ancient (uncalibrated dates) and present-day Eurasian samples. Red dots represent the archaeological sites sampled for ancient mitochondrial DNA in this study: aUZ, Yuzhnyy Oleni Ostrov; aPo, Popovo; aBOO, Bol'shoy Oleni Ostrov [65].

In [65] there is given a genetic argument for the relativeness of 'red' population (in sense of image 6) and the population of Siberia: «*The high frequency of hg U4 is a feature shared between Mesolithic aUzPo, present-day Volga-Ural Basin (VUB) (Komi, Chuvashes, Mari), and West Siberian populations (Kets, Selkups, Mansi, Khants, Nenets), with the latter group also being characterized, like aUzPo, by the presence of hg C*». And here we should remember that «***On the other hand, the Corded Ware sample, also from east-central Germany, is sitting at the other end of the plot, among groups from the Volga-Ural region***»

Mitochondrial haplogroup C, besides the Mesolithic Age of Karelia has been recently discovered in the population of Dnieper-Donets cultural community and even in the Bronze Age of the same region [**60, p 77**]. The presence of the haplogroup C in these districts, essential for the history of the corded ornament in the East Europe in the relevant period of time indicates the earlier migrations to this territory from the East (Mesolithic Age of Finland and Karelia is simultaneous with the Early Neolithic Age of the Ukraine).

One more possible marker of the migration from the regions, where the corded ornament has formed much earlier, than in the East Europe, is certainly Y-DNA haplogroup N and though, as it was mentioned above, it possibly has no concern with the bearers of the corded ware in Yakutia, its

presence is possible in the other center of this technique in the Baikal Region. Despite the Mesolithic migration of the C1f carriers, this migration could refer to the Neolithic Age, or Mezolithic latter than that of C. To trace and date these migration we can use data on mtDNAs. The migration of mt-haplogroup Z is notable among the mitochondrial haplogroups: «*On the basis of modern-day genetic data, hg Z1was proposed to have been introduced into populations of the VUB and Saami by migrations from Siberia via the southern Urals to the Pechora and Vychegda basins (northwest Urals), associated with the appearance of the* ***Kama culture ~8,000 yBP****. The presence of hg Z1 in aBOO establishes a direct genetic link between aBOO and modern-day populations of the VUB and Saami, and possibly indicates the trajectory of the migration that brought 'Central/East Siberian' lineages into NEE. The fact that aBOO did not contain any other Saami-specific haplotypes, suggests an independent origin and contribution of Z1 to the Saami gene pool*» [65].

We can notice that Z is present in the Eastern Siberia and completely absent in Americas: «*Thus, these six transitions are characteristic of the ancestral haplogroup Z haplotype. Based on the distribution of haplogroups C, S, Z and the number of variants encompasses, we postulate that southcentral Siberia was the place of origin for the ancestral haplotype, and the populations bearing haplogroup Z must have spread into northern Eurasia after the ancestors of the Amerindians left the region.*

Early studies of Native American mtDNA variation have shown that all Native American mtDNAs belong to haplogroups A, B, C, D and X, and that some of these haplogroups are also common along the northern Pacific Rim (Torroni et al. 1993a, b; Ward et al. 1993; Forster et al. 1996; Starikovskaya et al. 1998; Brown et al. 1998; Schurr et al. 1999). Specifically, the analysis of mtDNA diversity in the Chukchi and Siberian Eskimos of extreme northeastern Siberia revealed haplogroups A, C, and D. In contrast, the Koriak and Itelmen of the adjacent Kamchatka Peninsula, who speak a language from the same language phylum (Chukchi-Kamchatkan), harbour the east Eurasian haplogroups G, Z and Y, which are completely absent in Native Americans» [**63, p 10**].

The Kama culture could be the Early Neolithic bridge for the spread of mtDNA Z from the Far East (where it is common for the peoples of Korea and North China nowadays) to the west. May be this migration occurred together with the one, marked by Y-haplogroup N. **The simultaneous character of their occurrence and the absence in America point at it**. But neither classic corded, nor pseudo-corded wares haven't been discovered in the Kama culture yet [**17, p 243**], though the Kama culture has definite technological links with the South Trans-Ural in the Early Neolithic Age – primarily, with the Poludenovskaya culture [ibid**, p 247**].

Now it is to early to give a clear answer whether the Corded Ware ceramics was brought to the Eastern Europe from the East of not, and if it was so, which mtDNA's and Y-DNA'sreflected the migrations. But the argument of absence of (pseudo) corded Ware in Kamskaya culture allows to give some credit to the version of mtDNA C bearers as the people who could brought the Corded Ware style to Europe. Maybe htat migration was even accompanied with migration of a certain subclade of Y-DNA R1. The data on paleo mt-haplogroup C in the Eastern Europe is presented in table 1.

Table 1: Paleo mtDNA haplogroup C in the Eastern Europe

Time	**Region**	**Subclade**
6000 B. C.	Hungary	C5
5500 B. C.	Karelia	C1f
5400 B. C.	Ukraine	C
4000 B. C.	Ukraine	C4a2, C4a3, C4a4
3740 B. C..	Ukraine	C4a6
2000 B. C.	Ukraine	C4a3, C4a6

Sources: [**60, 61, 62]**. (The initial tables are given in supplement 2 and 3)

In [62] is clearly stated that «*East Eurasian lineages were represented by the C clade (Ya34 and Ya45), which is uncommon in ancient or present-day European populations, but is found in Neolithic populations, as well as contemporary populations from South Siberia, where this lineage is most likely originated (Starikovskaya et al., 2005; Mooder et al., 2006). Of interest in this context is the fact that the analysis of Neolithic cemeteries of the Baikal region has suggested that a depopulation event occurred in that region during the 6th millennium BP (Mooder et al., 2006).* ***As such, the dating of Yasinovatka (at ca. 6440–6080 [Hedges et al., 1995]) suggests that there is a possible link between the Baikal depopulation event and the appearance of the C lineage of mtDNA in the North Pontic region****. Whilst the analysis is limited in terms of the numbers of individuals analysed to date, it is of some interest that individual Yas19 (female 20–25) has been identified by Potekhina as being of local origin, and the U3 clade is linked to the founder haplotypes that probably entered Europe from the Near East or Caucasus, possibly linked to the LUP population expansion. A detailed report of the results of the genetic analysis of these specimens is in preparation at the time of writing*».

The mentioned association of the Baikal Region with the Dnieper Region, which is very important because of its possible influence on the Corded Ware origin in the East Europe, can be the searched paleogenetic link between the east and the west of Eurasia via the subclade C. The dating of the abovementioned migration is the other challenge. We can speak of the IV, maximum late V millennia BC.

If we return to the Dnieper Region, where subclades C were discovered from the Neolithic to the Bronze Ages, the Corded Ware could be found there in the Eneolithic Age. According to Merpert: «*Nevertheless, the definite role of the influence of the steppe tribes, who demonstrate the very early spread of the corded ornament, proved as the result of the researches of the recent years is highly possible*" and further: «*I don't mean the excavations of the wonderful Eneolithic settlement near Selo Derievki in the mouth of the Omel'nik River (the right confluent of the Dnepr) in Kirovograd Oblast by D.Ya.Telegin* (D.Ya. Telegin. From the Work of Dnepro-Dzerzhinsk Expedition of 1960 — Brief Reports of the Institute of Archaeology, Academy of Sciences of the Ukrainian Soviet Socialist Republic, issue 12. Kiev, 1962, p. 13-17)». The rich collection of the ware of this settlement, belonging to the pre-ancient-Yamnaya culture time of the forest-steppe Ukraine contains abundant and diverse examples of the corded ornament. This complex can be justly considered the oldest of the known «corded ware» complexes of Europe [**64, p 18**]. As subclades C were found near Derievka, it can be interpreted as another argument in favor of the Baikal-Dnepr migration.

There is one more hint of that the mito-haplogroup C got into the Derievka Region together with the carriers of some subclade Y-DNA R1. The case is that if we accept the theory that the carriers of Y-haplogroup R1 and the mt DNA haplogroup C one went from the Siberia epicenter of migration both to the West and to the East, the presence/absence of other haplogroups could provide more arguments. In other words, those haplogroups, which spread is correlated by some subclades of the analyzed one. And there are such results for one of the C subclade: «*An abstract in a 2012 issue of the "American Journal of Physical Anthropology" states that "The similarities in ages and geographical distributions for C4c and the previously analyzed X2a lineage provide support to the scenario of a dual origin for Paleo-Indians. Taking into account that C4c is deeply rooted in the Asian portion of the mtDNA phylogeny and is indubitably of Asian origin, the finding that C4c and* ***X2a*** *are characterized by parallel genetic histories definitively dismisses the controversial hypothesis of an Atlantic glacial entry route into North America*."[66].

Concerning mtDNA X the sub-group X2 appears to have undergone extensive population expansion and dispersal around or soon after the last glacial maximum, about 21,000 years ago. It is more strongly present in the Near East, the Caucasus, and Mediterranean Europe; and somewhat less strongly present in the rest of Europe. Particular concentrations appear in Georgia (8%), the Orkney Islands (in Scotland) (7%), and amongst the Israeli Druze community (27%). Subclades **X2a** and X2g are found in North America, but are not present in native South Americans.The New World haplogroup X2a is as different from any of the Old World X2b, X2c, X2d, X2e, and X2f lineages as they are from each other, indicating an early origin «*likely at the very beginning of their expansion and spread from the Near East*».[3] Although it occurs only at a frequency of about 3% for the total current indigenous population of the Americas**, it is a bigger haplogroup in the North America, where among the Algonquian peoples it comprises**

up to 25% of mtDNA types. It is also present in lesser percentages to the west and south of this area—among the Sioux (15%), the Nuu-Chah-Nulth (11%–13%), the Navajo (7%), and the Yakama (5%). Algonquian people (esp Ojibwe) became famous for the presense of a big share of R1 subclade. **R1 (M173)** is found predominantly in North American groups likethe Ojibwe (79%), Chipewyan (62%), Seminole (50%), Cherokee (47%), Dogrib (40%), and Tohono O'odham (Papago) (38%) [67]. This data gives the background for the hypothesis that as some Algonquin people has big percentages of mtDNA X and Y DNA R1, and mt DNA X is accompanied by mtDNA C, the mutual migration (or the start of migration or micromigration) of bearers of some subclades of mtDNA X, mtDNA C, Y-DNA R1. Some of them could have connections with Belkachinskaya and Syalakhskaya cultures.

So, the facts outlined in the paper give a certain basis to the hypothesis of the Far Eastern origins of the Corded Ware technology, and if that is true, Corded Ware could be brought to the Eastern Europe by a population which possessed mtDNA C and Y-DNA R1. And of course more paleogenetic and archaeological research of Eastern Europe, Ural, West and East Siberia should be carried out. The ultimate conclusion may be drawn only after the obtaining and carefully analysis of the statistics of the paleo-DNA data.

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SUPPLEMENT 1
http://polishgenes.blogspot.com.au/2015/06/badasses-of-bronze-age-analysis-of.html

...But we already have a reasonable collection of ancient DNA from the relevant archeological cultures. Does it back the general consensus? Let's take a look, starting with the Y-chromosome data sorted by culture. The bracketed numbers are the sample sources, which are listed at the bottom of the post.

Corded Ware, Germany, Individuals 2,3,4 *[1]*, **R1a**
Corded Ware, Germany, I0104 *[3]*, **R1a**
Corded Ware, Germany, RISE434 *[4]*, **R1a**
Corded Ware, Germany, RISE436 *[4]*, **R1a**
Corded Ware, Poland, RISE1 *[4]*, **R1b?**
Corded Ware, Germany, RISE446 *[4]*, **R1a**
Corded Ware, Poland, RISE431 *[4]*, **R1a**

Single Grave?, Denmark, RISE61 *[4]*, **R1a**

Battle-Axe, Sweden, RISE94 *[4]*, **R1a**
Battle-Axe?, Sweden, RISE98 *[4]*, **R1b**

Sintashta, Trans-Urals, Russia, RISE386 *[4]*, **R1a**
Sintashta, Trans-Urals, Russia, RISE392 *[4]*, **R1a**

Andronovo, South Siberia, Russia, S07 *[2]*, **C**
Andronovo, South Siberia, Russia, S10 *[2]*, **R1a**
Andronovo, South Siberia, Russia, S16 *[2]*, **R1a**
Andronovo, Altai region, Russia, RISE512 *[4]*, **R1a**

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SUPPLEMENT 2

Table 2 | Individual life history data for selected individuals from Yasinovatka as based on the mtDNA analysis

Cemetery	Individual	Sex/Age	Chronological Age (calBC)	Clade/haplogroup	Likely origin of the haplogroup
Yasinovatka	Yas54	♂ 30–40	5616–5482	T	Near East/West Eurasia
"	Yas19	♀ 20–25	5434–5221	U3	Near East/Caucasus
"	Yas17	adultus	5437–5090	Likely U1	West Eurasia
"	Yas45	♂ 20–25	5432–5148	C	East Eurasia
"	Yas34	infantilis	ND (not-dated)	C	East Eurasia
"	Yas32	♂ 30–40	ND (not-dated)	T	Near East/West Eurasia

Source: **[62], p 88.**

SUPPLEMENT 3

Table 3. MtDNA HVSI sequences of Neolithic and Bronze Age individuals from the North Pontic Region.

Sample	Age (BP)	Time Period	HVSI Sequence (+16000) [c, d]	RFLPs (where available)	Haplogroup
Ni58	2,305±45[b]	Neolithic	061, 223, 298, 327		C
Ya34	6,195±80	Neolithic	223, 298, 327, 357		C4a2'3'4'
Ya45	6,360±60	Neolithic	223, 298, 327		C
D1.8	3,940±70	Bronze Age	223, 278, 298, 327, 357		C4a3
L8	3,990±70	Bronze Age	218, 223, 288, 298, 305A-T, 327, 357		C4a6
L15	3,740±70	Bronze Age	218, 223, 298, 327, 357		C4a6
DD33	6,175±60	Neolithic	311	-7025AluI	H
DD38	N/A	Neolithic	*080*	-7025AluI	H
Ya17	6,360±75	Neolithic	241		H? [e]
Ya36	6,260±180	Neolithic	320		H? [e]
Ya57	N/A	Neolithic	241	-7025AluI	H
Ya64	6,330±90	Neolithic	064, 240	-7025AluI	H
Ya32	4,500±120	Neolithic	*294, 296*		T
Ya54	6,593±35	Neolithic	*294, 296*		T
Ni79	5,200±30	Neolithic	*294, 296*		T
Ya19	6,370±60	Neolithic	343		U3
Ni94	6,225±75	Neolithic	256, 270, 356		U5a

[a] Ya – Yasinkovatka; DD – Dereivka; Ni – Nikolskoye;
[b] The radiocarbon date for this sample is likely inaccurate (see text for explanation)
[c] All mutations listed are transitions compared to the rCRS, unless noted explicitly.
[d] Italics denote incomplete HVSI sequences.
[e] RFLP status could not be determined for these samples.

Source: [60].