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Molecular Markers: an Introduction and Applications

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Abstract

The dramatic development of molecular genetics has laid the groundwork for genomics. It has introduced new generations of molecular markers for use in the genetic improvement of farm animals. These markers provide more accurate genetic information and better understanding of the animal genetic resources. Scientists, unfamiliar with the different molecular techniques tend to get lost as each has its own advantages and disadvantages. This review represents a trail to shade alight on the different types of molecular markers by introducing a brief summary on the development of genetic markers including both the classical genetic markers and more advanced DNA-based molecular markers. This review could be helpful to better understand the characteristics of different genetic markers and the genetic diversity of animal genetic resources.

Keywords: SNP, microsatellite, molecular marker, genome, polymorphism.

Introduction

A genetic marker is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like mini & micro satellites.

Recent years have witnessed a great interest towards molecular markers, revealing polymorphism at the DNA level, as they play an important role in animal genetics studies. Sometimes the term “Smart Breeding” is used to describe marker supported breeding strategies.

The main aim of the breeder is to select animal with superior genetic potential as parents for the next generation. The first attempt to improve animals used the phenotype of an animal for a specific trait as a tool for selection. This application uses external animal characteristics as a marker that called morphological markers (i.e. udder shape, coat color, body shape, skin structure, and anatomical characteristics) [1, 2]. These markers depend on visual observation and measurement to identify, classify, and characterize the genetic evolution of different species or populations. The conclusions reached through applying morphological markers are often not completely accurate when they used for the evaluation of farm animal genetics, because these markers based on subjective judgments and descriptions. Another type of markers represents by using of cytological markers that were included several criteria such as chromosome karyotypes, bandings, repeats, translocations, deletions, and inversions to investigate the genetic resources of

animals [3]. The chromosome mutations lead to genetic variation [4]. These mutations were used as markers to identify a certain location of the gene on a specific chromosome. In the domestic animals, cytological markers allow to investigate their genetic diversity by comparing the chromosome number and the structure between domesticated animals and their wild ancestors [5]. Cytological markers are still widely used in elucidating the origin and classification of species [6] because of their good properties, rapid economic and straightforward technique.

The third type of markers is biochemical markers, such as the blood type and isozymes. These markers represent biochemical traits that could be analyzed by protein electrophoresis. The differences in the amino acid composition of isozymes and soluble proteins were used to investigate the genetic variation within species and phylogenetic relationships between species [7]. The application of these markers was limited because the proteins and isozymes are not genetic materials. They are products of gene expression, so they could be affected by environmental factors [8]. Thus, the direction attention of researchers is converted to the molecular markers. The molecular markers are based on the nucleotide sequence mutations within the individual's genome; they are the most reliable markers available [3].

Marker assisted selection (MAS)

Selection is one of the most important tools to improve the performance of animals. It can be accomplished based on two types of data – pedigrees and phenotypes to estimate Best Linear Unbiased Prediction (BLUP) that combines these to generate estimated breeding values (EBVs). A third type of data is based on DNA markers to get a new approach named Marker assisted selection (MAS). The MAS can be based on DNA in linkage equilibrium with a quantitative trait locus (QTL) (LE-MAS)–LE refers to genotype frequencies at one locus are independent of genotype frequencies at the second locus, - molecular markers in linkage disequilibrium with a QTL (LD-MAS)–LD refers to the non-random association of alleles between two loci-, or based on selection of the actual mutation causing the QTL effect (Gene-MAS). All three types of MAS are being used in the livestock industries [9].

Molecular and quantitative genetics

The most economically important traits in livestock are quantitative, that they show continuous distributions. Two models have been proposed to explain the genetic variation among such traits, the infinitesimal model (the basis of quantitative genetics) and the finite loci model (the basis of molecular genetics). The infinitesimal model assumes that traits are determined by an infinite number of unlinked and additive loci, each with an infinitesimally small effect [10]. This model has been exceptionally valuable for animal breeding, and forms the basis for the breeding value estimation theory [11]. The finite loci model assumes the existence of a finite amount of genetically inherited material (the genome). There are a total of around 20000 genes or loci in the genome [12]. Many evidences confirmed that the distribution of the effect of these loci on quantitative traits could be classified to a few genes with large effect and a many of small effect [13, 14]. The search for these loci, particularly those of moderate to large effect, and the use of this information to increase the accuracy of selecting genetically superior animals, has been subjected to intensive research studies in the last two decades.

The first approach of applying molecular markers has been used is the candidate gene. It is assume a gene involved in a certain trait could show a mutation causing variation in that trait, and any variations in the DNA sequences, that are found, are tested for association with variation in the phenotypic trait [15]. Although this approach has achieved some success – for example a mutation that discovered in the estrogen receptor locus (ESR) which results in the increased litter size in pigs [16], but two problems have faced this approach. Firstly, candidate genes affecting a trait usually have a large number, so many genes must be sequenced and a large sample of animals is needed. Thus, the likelihood that the mutation may occur in non-coding DNA further increases the amount of sequencing required. Secondly, the mutation that associated with the phenotypic variation in a certain trait could occur in another gene that considered a non-candidate gene.

Up to now, many types of molecular markers have been utilized to detect the variation among individual and population. These markers can be classified into three groups; protein variants (allozymes), DNA sequencepolymorphism, DNA repeat variation. So it is very necessary to conduct a review on most important molecular markers.

Allozyme markers

Allozymes are enzyme variants due to allelic differences and can be visualized through protein electrophoresis. This technique was developed to quantify the genetic and geographic variation in wildlife populations, and it remains a cost-effective and straightforward method [17]. Genetic variations caused by mutations are expressed as amino acid replacements due to changes in protein compositions, and are resolved as bands (alleles) on electrophoretic gels [18].

These markers provided a valuable tool for population genetic studies in natural populations of woody plants [19]. They usually exhibit simple Mendelian inheritance and codominant expression, making genetic interpretations easy. In addition, allozyme analysis is relatively fast, inexpensive, and an extensive literature exists about it [20, 21]. However, allozymes have limitations, such as highly biased genomic sampling (only genes encoding well-documented, soluble proteins are detectable); a low number of markers, insufficient for examining major portions of the genome; occasional differences between tissues or ontogenetic stages; and difficulty in the standardization of experimental methods from laboratory to laboratory.

Mitochondrial DNA (mtDNA)

mtDNA is an extra-chromosomal genome in the cell mitochondria that resides outside of the nucleus, and is inherited from mother with no paternal contribution [17]. Due to higher evolutionary rates of mtDNA relative to the nuclear genome [22], this marker is preferred in constructing phylogenies and inferring evolutionary history, and is therefore, ideal for within- and between-species comparisons [18]. The drawbacks of mtDNA analyses include hybridization, introgression, and incomplete lineage sorting. Moreover, mtDNA is of little use in investigating the recent loss of genetic variation and any individual-level events such as identity, individual dispersal, and mating systems [22].

Restriction Fragment Length Polymorphism (RFLP)

The RFLP is a technique that is not widely used now, but it was one of the first techniques used for DNA analysis in forensic science and several other fields. The RFLP is defined by the existence of alternative alleles associated with restriction fragments that differ in size from each other.

The molecular basis of the RFLP is that nucleotide base substitutions, insertions, deletions, duplications, and inversions within the whole genome can remove or create new restriction sites [3].

Despite the fact that it is less widely used now, there have been numerous benefits to RFLP analysis. It plays an important role in allowing scientists to map the human genome as well as provide information on genetic diseases [23]. RFLP analysis is useful to find out where a specific gene for a disease lies on a chromosome. This is performed by looking at the DNA from a set of family members who suffer from a certain disease and then searching for RFLP alleles that share the same type of inheritance pattern for the condition. Using RFLP analysis, enable scientists to determine others who might be at risk for the disease or a carrier of the mutated gene.

The RFLP was also one of the first methods used for genetic typing - also known as genetic fingerprinting, profiling or testing. Despite that the RFLP have many benefits but it is still a slow and more tedious process compared to some of the newer DNA analysis techniques. Ii is also requires substantially larger sample sizes than other forms of analysis.

Random Amplification of Polymorphic DNA (RAPD):

In the last decade, the RAPD technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers [24].

The RAPD technology provides a quick and efficient screen for DNA sequence based polymorphism at a very large number of loci. The major advantage of RAPD includes that, it does not require pre-sequencing of DNA [25].

The RAPD analysis has been extensively used for various purposes which include identification and classification of accessions [26], identification of breeds [27] and genetic diversity analysis [28].

The principle of RAPD is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that

the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome [25]. Since the advantages of RAPDs are the technical simplicity and the independence of any prior DNA sequence information, [29, 30] it is viewed as having several advantages compared to RFLP and fingerprint [31].

A disadvantage of RAPD markers is the fact that the polymorphisms are detected only as the presence or absence of a band of a certain molecular weight, with no information on heterozygosity besides being dominantly inherited, and also show some problems with reproducibility of data [32].

Amplified Fragment Length Polymorphism (AFLP)

AFLP markers have found the widest application in analyses of genetic variation below the species level, particularly in investigations of population structure and differentiation [33].

AFLP methods rapidly generate hundreds of highly replicable markers from DNA; thus, they allow high-resolution genotyping of fingerprinting quality. The time and cost efficiency, reproducibility and resolution of AFLPs are superior or equal to those of other markers (RAPD, RFLP and microsatellites) [32]. AFLP markers have emerged as a major new type of genetic marker with broad application in systematic, pathotyping, population genetics, DNA fingerprinting and quantitative trait loci (QTL) mapping [34].

However, AFLPs are dominant bi-allelic markers, [35] and are unable to distinguish dominant homozygous from dominant heterozygous individuals [36]. The AFLP method is an ideal molecular approach for population genetics and genome typing, it is consequently widely applied to detect genetic polymorphisms, evaluate, and characterize animal genetic resources [37, 38, 39, 40].

Microsatellites

Microsatellites or simple sequence repeated (SSR) loci, which have been referred to in the literature as variable number of tandem repeats (VNTRs) and simple sequence length polymorphisms (SSLPs), are found throughout the nuclear genomes of most eukaryotes and to a lesser extent in prokaryotes [41, 42].

Microsatellites range from one to six nucleotides in length [43] and are classified as mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats. The sequences of di-, tri- and tetranucleotide repeats are the most common choices for molecular genetic studies [44]. They are tandemly repeated (usually 5-20 times) in the genome with a minimum repeat length of 12 base-pairs [45, 46, 47].

The number of repeats is variable in populations of DNA and within the alleles of an individual. The sequence below has a 20 dinucleotide repeat (40bp) stretch of CA that is shown in **bold**.

CGTTCAATAAGCAAAAATCCATAGTTTTAGGAATGTGGGCT
GCTTGGTGTGATGTAGAAGGCGCCAATGCATCTCGACGTAT
GCGTATACGGGTTACCCCCTTTGCAATCAGTG**CACACACAC**
ACACACACACACACACACACACACACACACACAGTGCCAAGCA
AAAATAACGCCAAGCAGAACGAAGACGTTCTCGAGAACACC
AGAAGTTCGTGCTGTCGGGGCATGCGGCGAGTAAAGGGGAT

When a microsatellite flanked with fluorescent PCR primers then the amplification will give a pair of fluorescent allelic products which will vary in size according to their repeat length. A population might possess 5 alleles which vary in size as illustrated in Fig 1.

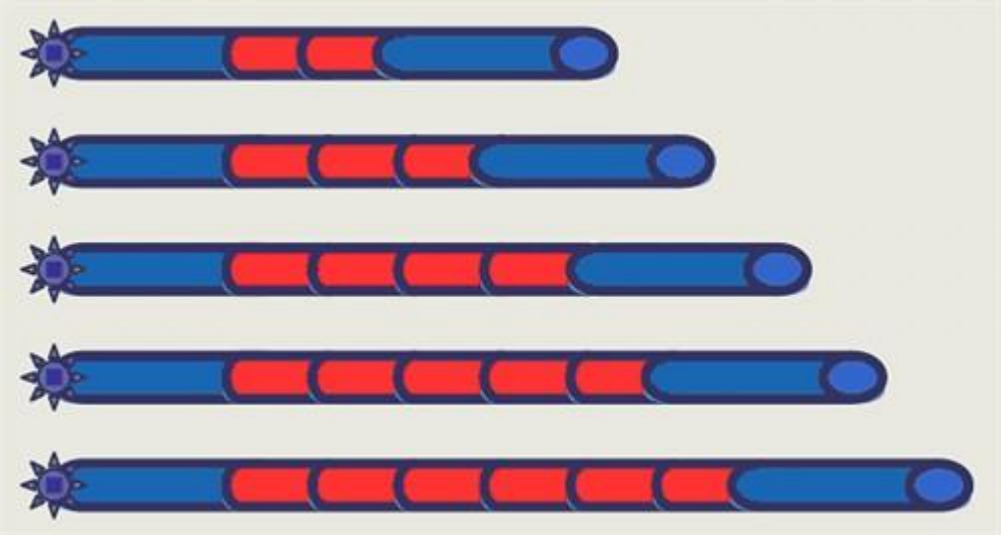

Figure 1: Five Alleles with Different Repeat Length

Microsatellites can be used as markers in genetic studies of linkage in families and linkage disequilibrium studies of populations. In linkage studies one can examine large number of families and see when the alleles of specific markers are inherited together with a phenotype in more cases than not. Microsatellite repeat are amplified with fluorescently labeled primers and then the alleles from each individual in a family are separated by size and the marker tested for linkage with another as shown in Figure 2.

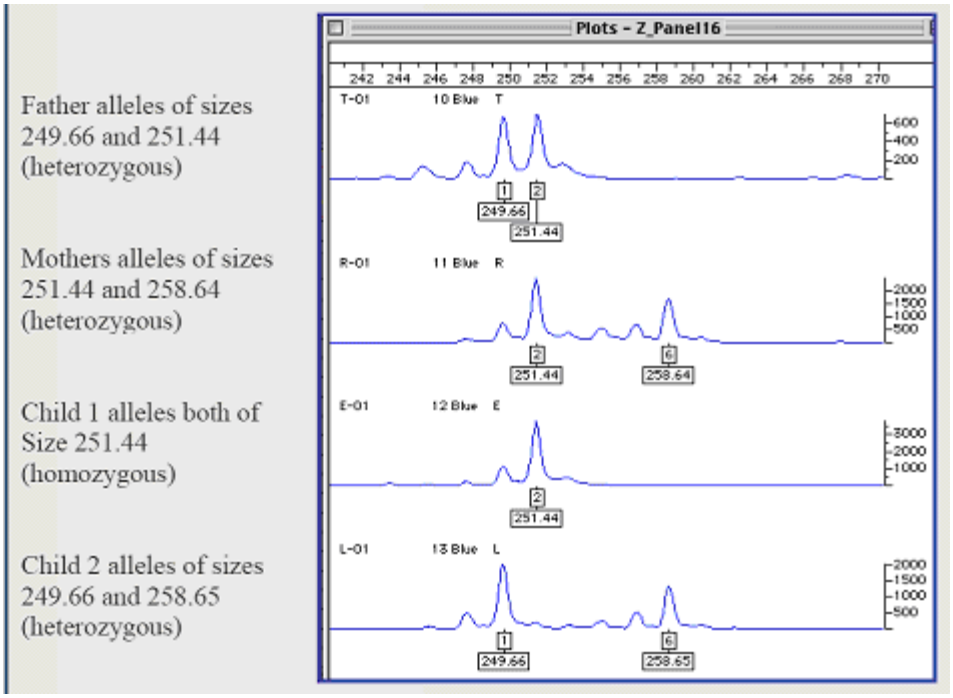

Figure 2: Raw of Genotyping Data

This approach assumes that a certain quantitative trait was affected by many unknown genes. So, this approach is looking for associations between the variation of allele and quantitative traits at the neutral DNA markers. The DNA marker is located on a chromosome and its inheritance can be monitored [48].

Microsatellites are the most commonly applied molecular marker in ecological research (Fig. 3).

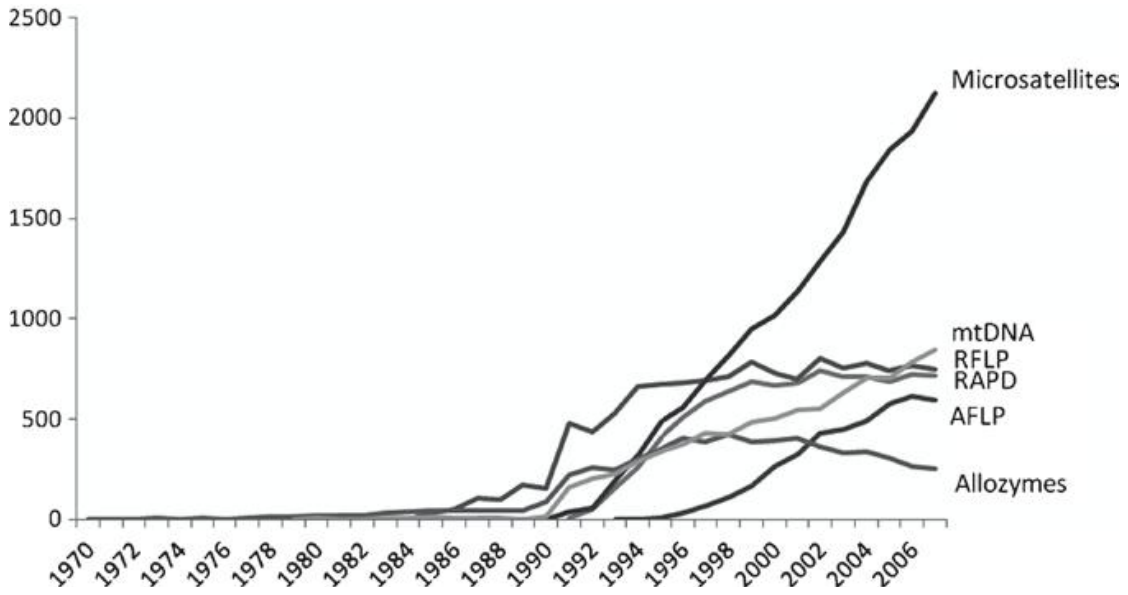

Figure 3: Number of publications (selected biological subject) between 1970 and 2007 employing mtDNA, Allozymes, Microsatellites, RFLPs, RAPDs and AFLPs found via ISI web of knowledge

Types of microsatellites

The types of SSRs could be classified based on:

1- Occurrence and source for development:

a. Genomic or nuclear microsatellites (gSSRs) - microsatellites isolated from the nuclear genome (genomic DNA of an organism with or without the construction of genomic DNA library).

b. EST or genic microsatellites (EST-SSRs) - microsatellites developed by data-mining or exploiting EST sequences deposited in public databases.

c. Organellar microsatellites [chloroplast SSRs (cpSSRs) and mitochondrial SSRs (mtSSRs)] - microsatellites developed from the chloroplast or mitochondrial genome of an organism.

2- The type of repeat sequence [49]:

a. Simple perfect - the repeat sequence is continuous and is not interrupted by any base not belonging to the motif [e.g. AGAGAGAGAGAG or (AG)6].

b. Simple imperfect - the arrays consist of one or more repeat units of different lengths [e.g. AGAGAGAGAGCTAGAGAG or (AG)5CT(AG)3].

c. Compound-perfect - the arrays are composed of two or more different repeat motifs of the same length [e.g. AGAGAGAGCGTGAGAGAGAG or (AG)4CGTG(AG)4].

d. Compound imperfect - motifs are interrupted by one or more repeats of different length [e.g. AGAGAGAGAGTCTCTCTC or (AG)5(TC)4].

3 - The length of repeat motif [50]:

a. Class I microsatellites- perfect SSRs of ≥20 nucleotides in length.

b. Class II microsatellites- perfect SSRs of ≥12 nucleotides and ≤20 nucleotides in length.

Microsatellite markers have several advantages as they are considered to be robust [51] and more variable and informative than RFLP, RAPD [52] and AFLPs [53]. Using the technique of PCR-based require only low quantities of template DNA [54, 55]. Moreover, these markers are considered to be the best marker system for the detection of intervarietal polymorphisms [56]. They are also useful for parentage analysis and for estimating the degree of relatedness of individuals or groups [51]. On the other hand these markers have several disadvantages: expensive, laborious and time-consuming [57, 58, 59]. The low frequency of SSRs in plants also hinders the large scale isolation of SSRs [60].

Homoplasy is another problem when applying microsatellites as a reliable tool for phylogenetic analysis because alleles considered to be identical in state are not necessarily identical by descent [61].

Single-nucleotide polymorphism (SNP)

In 1996, Lander proposed a new molecular marker technology named SNP. When a single nucleotide (A, T, C, or G) in the genome sequence is altered this will represent the SNP (Figure 3). In other words, it refers to a sequence polymorphism caused by a single nucleotide mutation at a specific locus in the DNA sequence [3].

This sort of polymorphism includes single base transitions, transversions, insertions and deletions [41], and the least frequent allele should have a frequency of 1% or greater [62]. Transitions are the most common (approx. 2/3) among all the SNP mutation types [63], as shown in Figure 4. Currently, SNP markers are one of the popular approach, despite they can be considered as a step backwards (simple bi-allelic co-dominant markers) when compared to the highly informative multi-allelic microsatellites. This popularity of the SNPs based on some preferred properties; they are abundant in the genome, genetically stable, and amenable to high throughput automated analysis [64]. The more recent SNP concept has basically arisen from the recent need for very high densities of genetic markers for the studies of multifactorial diseases [64].

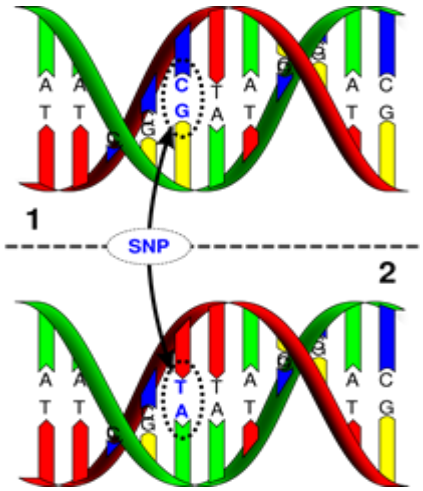

Figure 4: SNPs in DNA

The fundamental principle of SNPs is to hybridize detected DNA fragments with high-density DNA probe arrays (also called SNP chips); the SNP allele is then named according to the hybridization results [3].

SNPs are third generation molecular marker technology coming after RFLPs and SSRs [65]; it was successfully performed to investigate genetic variation among different species and breeds [66-68].

The role of SNPs in farm animals was very important concerning the population structure, genetic differentiation, origin, and evolution research [3]. On the other hand, the most important disadvantage of SNPs is the low level information obtained as compared with that of a highly polymorphic microsatellite but this can be solved by using a higher numbers of markers (SNP chips) and whole-genome sequencing [69-70].

Variations in the DNA sequences of humans can affect how humans develop diseases; respond to pathogens, chemicals, drugs, etc. However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts (such as with matched cohorts with and without disease. The study of single nucleotide polymorphisms is also important in crop and livestock breeding programs.

DNA barcoding markers

A DNA barcode is a short DNA sequence from a standardized region of the genome used for identifying species. The essential aim of DNA barcoding is to use a large-scale screening of one or more reference genes in order to assign unknown individuals to species, and to enhance discovery of new species [71-72].

Biological taxonomists apply this principle to species classification. The first application of using the DNA sequences in systematical biological taxonomy (also called DNA taxonomy) was conducted by Tautz et al. [73] and then, Hebert et al. [71] proposed the concept of DNA barcoding and suggested its use for a single mtDNA gene, mitochondrial cytochrome *c* oxidase I (COI), as a common sequence in animal DNA barcoding studies.

The DNA barcoding has a high accuracy of 97.9% [59], and provides a new, quick, and convenient identification strategy for animal genetic diversity [74]. This approach like previous mentioned markers have some disadvantages represents by the genome fragments are very difficult to obtain and are relatively conservative and have no enough variations. Some organisms cannot be identified with COI because of the low evolution rates of COI sequences in some species. Moreover, COI is an mtDNA sequence of maternal origin, which could bias species diversity [75, 76].

Conclusion

The accurate genetic evaluation of animals is the primary target for their conservation and utilization. Different methods have been developed and tested at the DNA sequence level. These methods provide a large number of markers and opening up new opportunities for evaluating diversity in farm animal genetic resources. Among all these methods, microsatellites remained the marker of choice for the past 15 years [77-80], due to their highly polymorphic and hence informative nature [81].

However, due to their complex and varied mutational patterns, as well as high genotyping error rates and relatively low density throughout the genome, they have recently received much scrutiny [82, 83]. More specifically, their application in estimating genetic diversity within and between populations was recently challenged by Väli et al. [82] after demonstrating that multilocus heterozygosity does not reflect genome-wide diversity, reinforcing a similar perspective initially highlighted over 30 years ago [84, 85]. The authors suggested instead that SNPs to get a more accurate means of assessing overall genomic diversity in natural populations.

Currently, SSR and SNP markers subjected to many researches to compare their validation for map built.

In a simulations prediction study [77], results showed that SNPs are at least two to six times more necessary to achieve the same resolution as microsatellites when used for individual identification and the study of parentage assessment and relatedness.

The SNP markers have promising advantages over microsatellite markers, due to high-throughput automated analysis, lower mutation rates and lower genotyping costs [77, 86].

In a study conducted by Ball et al. [87] a comparison among linkage maps built with microsatellites, SNPs and a combination of both markers was done to determine the ability of each method to produce accurate linkage maps. Results revealed that, although microsatellites are informative, they can provide misleading results because they have greater error rates than SNPs. The potential inflation in map and incorrect marker orders associated with the microsatellite genotyping led the Ball et al., [87] to formulate a conclusion as "we are not suggesting that microsatellites should be abandoned in mapping studies, but we do urge that appropriate error checking precautions are employed".

Defaveri et al. [88] stated that; for microsatellites, there is a standard procedure for genotyping involving the PCR and size determination of the amplified fragment by gel electrophoresis. Whereas, no standard method for analysis must be used in SNP genotyping and many techniques are available [64, 86].

Their results also confirmed that the genomic location of markers had a strong impact on the results generated from microsatellites, but not on those from SNPs. Results also reinforce the perspective that microsatellite variability does not reflect genome-wide diversity [82], and that this genomic heterogeneity can affect the estimates of demographic and selective forces acting in wild populations. However, some reports on humans [89] and plants [90], showed that the SNP markers can only be transferred to different mapping populations within the same species, but not across species. In view of these results, Wang et al. [91] claimed this will limit the applications of SNP markers on related minor species. In contrast, due to multiple alleles, cost-effectiveness, and transferability, SSR markers will continue to play an important role in different genetic studies in many minor plant and insect species in the future.

The continuous development of molecular markers along with innovation of new statistical methods and the available of software could be end the debate about this subject by identification which of them is the best.

This certainly will lead to more progress in application of molecular markers in animal breeding.

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Possible Processes for Origin of First Chemoheterotrophic Microorganisms with Modeling of Physiological Processes of Bacterium *Bacillus subtilis* as a Model System in ^2H_2O

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Abstract

We studied possible processes for origin of first chemoheterotrophic microorganisms with modeling of physiological processes of a Gram-positive chemoheterotrophic bacterium *Bacillus subtilis*, producer of purine ribonucleoside inosine as a model system in heavy water. The physiological influence of deuterium on the chemoheterotrophic bacterium *B. subtilis* was studied on a heavy water (HW) medium with a maximal concentration of ^2H_2O (89–90 atom% ^2H). Also various suitable samples of hot mineral water and sea water derived from different sources of Bulgaria were investigated using IR- and DNES-spectroscopy. It was shown that hot alkaline mineral water with temperature from +65 °C to +95 °C and pH value from 9 to 11 is more suitable for the origination of first organic forms than other analyzed water samples. There were discussed the reactions of condensation and dehydration occurring in alkaline aqueous solutions at t = +65–95 °C and pH = 9–10, resulting in synthesis from separate molecules the larger organic molecules as short polipeptides and pyrines, as well as the possible mechanisms of the deuterium accumulation in form of H^2HO in hot water. The metabolism of the bacterium *B. subtilis* and the resistance to deuterium was also analyzed on an evolutionary level taking into account the hydrological conditions of primodial hydrosphere and the presence of H^2HO, as well as the qualitative and quantitative composition of the cellular protein, amino acids and carbohydrates on media with maximum deuterium content. It was demonstrated on the example of chemoheterotrophic bacteria that first microorganisms might have been originated in hot mineral water with Ca^{2+} (0.5-1.0 g/l) at t = + 65-95 °C and pH = 9–11, that is more suitable for maintenance and origin of life than other analyzed water samples.

Keywords: heavy water, glycolysis, purines, amino acids, *Bacillus subtilis*, hot mineral water, origin of life

Introduction

One of the most interesting biological phenomena is the ability of some microorganisms to grow in heavy water (HW) media in which all hydrogen atoms are replaced with deuterium (^2H) [1]. The chemical structure of the ^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. That difference stipulates the isotopic effects, which may be sufficiently big for H/^{2}H pair [2]. As a result, physical-chemical properties of ^2H_2O differ from H_2O: ^2H_2O boils at t = +101.44 ^oC, freezes at t = +3.82 ^oC, has maximal density at t = +11.2 ^oC (1.106 g/cm^3). The chemical reactions in ^2H_2O are somehow slower compared to H_2O. ^2H_2O is less ionized, the dissociation constant is smaller, and the solubility of the organic and inorganic substances in ^2H_2O is smaller compared to these ones in H_2O. Due to isotopic effects the hydrogen bonds with the participation of deuterium are slightly stronger than those ones formed of hydrogen. According to the theory of chemical bond, breaking up of covalent H–O-bonds can occur faster, than ^{2}H–O-bonds, mobility of $^2H_3O^+$ ion is lower on 28.5% than H_3O^+ ion, and O^2H^- ion – on 39.8% than OH^- ion. The maximum kinetic isotopic effect, which can be observed at ordinary temperatures in chemical reactions leading to rupture of bonds involving H and ^{2}H lies in the range of 4 to 6 for C–H versus C–^{2}H, N–H versus N–^{2}H, and O–H versus O–^{2}H-bonds [3].

These chemical-physical factors lead to slowing down in the rates of enzymatic reactions in ^2H_2O [4]. 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 ^{1}H with ^{2}H 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 [5]. Isotopic effects of deuterium, which would occur in macromolecules of even a small difference between hydrogen and deuterium, would certainly have the effect upon the structure. The sensitivity of enzyme function to the structure and the sensitivity of nucleic acid function (genetic and mitotic) would lead to a noticeable effect on the metabolic pathways and reproductive behavior of an organism in the presence of ^2H_2O [6]. 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.

The average ratio of $^2H/^1H$ in nature makes up approximately 1:5700 [7]. 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 ^{2}H 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 the SLAP standard isotopic shifts for ^{2}H 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 the 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 positive deviations at $2.0\cdot10^{-5}$. In mixtures of ^2H_2O with H_2O it is occurred with high speed the isotopic exchange with the formation of semi-heavy water (H^2HO): $^2H_2O + H_2O = H^2HO$. For this reason deuterium presents in smaller content in aqueous solutions in form of H^2HO, while in the higher content – in form of ^2H_2O.

For a long time it was considered that heavy water is incompatible with life. Experiments with the cultivation of cells of different organisms in ^2H_2O show toxic influence of deuterium. The high concentrations of ^2H_2O lead to the slowing down the cellular metabolism, mitotic inhibition in the prophase stage and in some cases – somatic mutations [8]. Experiments show that ^2H_2O influences negatively the different organisms. This is observed even while using natural water with an increased content of ^2H_2O or H^2HO [9]. Bacteria can endure up to 90% (v/v) ^2H_2O [10], plant cells can develop normally up to – 75% (v/v) ^2H_2O [11], and animal cells – up to not more than 35% (v/v) ^2H_2O [12]. The decrease of the deuterium content in water up to 25% (v/v) of the physiological level stimulates the cellular metabolism [13].

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 [14]. 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 green algae, which, being eukaryotes, are the more difficult adapted to D_2O and, therefore, exhibit inhibition of growth at 70–75 % (v/v) D_2O.

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 [15]. 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 [16].

This study is a continuation of our research for studying the possible processes for origin of first organic forms of life in various waters with varrying content of deuterium. The content of deuterium in hot mineral water may be increased due to the physical chemical processes of the deuterium accumulation. It can be presumed that primary water might contain more deuterium at early stages of evolution of first living structures, and deuterium was distributed non-uniformly in the hydrosphere and atmosphere [17]. The primary reductive atmosphere of the Earth consisted basically of gas mixture CO, H_2, N_2, NH_3, CH_4, lacked $O_2–O_3$ layer protecting the Earth surface from rigid short-wave solar radiation carrying huge energy capable to cause radiolysis and photolysis of water. The processes accompanying accumulation of deuterium in the hydrosphere are solar radiation, volcanic geothermal processes and electric discharges in the atmosphere. These natural processes could lead to the enrichment of the hydrosphere by deuterium in the form of H^2HO which evaporates more slowly than H_2O, and condenses faster. If this is true, this is a significant fact regarding thermal stability of deuterated macromolecules in the preservation of life under thermal conditions, because chemical bonds with participation of deuterium are somewhat stronger than those ones formed of hydrogen.

The object for study relate to different taxonomic groups of organisms having a chemoheterotrophic pathway of assimilation of carbon substrates as glucose via glycolysis. It is believed that the initial life forms on Earth had probably existed as chemoheterotrophic bacteria that received food and energy from organic substrates [18]. That is why the chemoheterotrophic bacterium *Bacillus subtilis* was chosen as a model for our studies.

The purpose of our research was studying the influence of deuterium on metabolic pathways of *B. subtilis* applicable to possible processes for origin of life and living matter.

Material and methods

Biological objects

The object of the research was a strain of inosine producer, spore-forming aerobic Gram-positive chemoheterotrophic bacterium *B. subtilis B-3157*, polyauxotrophic for histidine, tyrosine, adenine, and uracil (demand, 10 mg/l), obtained from Institute of Genetics and Selection of Industrial Microorganisms (Russia). The initial strain was adapted to deuterium by plating individual colonies onto 2% (w/v) agarose with stepwise increasing gradient of ^2H_2O concentration and subsequent selection of individual cell colonies stable to the action of ^2H_2O.

Water samples

The research by the IR-spectrometry (DNES-method) was carried out with samples of water taken from various water springs of Bulgaria:

1 – Mineral water (Rupite, Bulgaria);
2 – Seawater (Varna resort, Bulgaria);
3 – Mountain water (Teteven, Bulgaria);
5 – Deionized water (the control);
6 – Water with varying deuterium content (HDO).

As model systems were used cactus juice of *Echinopsis pachanoi*.

Chemicals

For preparation of growth media was ^2H_2O (99.9 atom% ^2H), ^2HCl (95.5 atom% ^2H), and [^2H]methanol (97.5 atom% ^2H), purchased from JSC "Izotop" (St. Petersburg, Russian Federation). 5-dimethylamino(naphthalene)-1-sulfonyl (dansyl chloride) of analytical reagent grade was from Sigma-Aldrich Corporation (St. Louis, USA). Deionized water was provided by the Milli-Q integral water purification system ("Millipore", USA). Inorganic salts and glucose ("Reanal", Hungary) were initially crystallized in 99.9 atom% ^2H_2O. ^2H_2O was distilled over $KMnO_4$ with subsequent control of the isotope purity by NMR spectroscopy on a Brucker WM-250 ("Brucker Corp.", USA) with a working frequency of 70 MHz (internal standard – Me_4Si).

Biosynthesis of ^2H-inosine

[^2H]inosine was produced with an output 3.9 g/l in heavy water (HW) medium (89–90 atom% ^2H) with 2% (w/v) hydrolysate of deuterated biomass of methanol-assimilating strain of the facultative Gram-negative methylotrophic bacterium *Brevibacterium methylicum* as a source of ^2H-labeled growth substrates. The strain was obtained by multistage adaptation on a solid 2% (w/w) agarose M9 minimal medium containing 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 0.5 g/l NaCl, and 1 g/l NH_4Cl with 2% (v/v) [^2H]methanol and a stepwise increasing ^2H_2O concentration gradient (0, 24.5, 73.5, and 98% (v/v) ^2H_2O). A raw methylotrophic biomass (yield, 200 g/l) was suspended in 100 ml 0.5 N ^2HCl (in ^2H_2O) and autoclaved for 30–40 min at 0.8 atm. The resulting suspension was neutralized with 0.2 N KOH (in ^2H_2O) to pH = 7.0, and used as a source of growth substrates when cultivating the inosine producer strain. For this purpose, an inoculum (5–6% (w/w)) was added to the HW medium with ^2H_2O containing 12% (w/w) glucose, 2% (w/w) hydrolysate of deuterated biomass *B. methylicum*, 2% (w/w) NH_4NO_3, 1% (w/w) $MgSO_4$ $7H_2O$, 2% (w/w) $CaCO_3$, 0.01% (w/w) adenine, and 0.01% (w/w) uracil. A protonated medium with 2% (w/w) yeast protein–vitamin concentrate (PVC) was used as a control.

Growth conditions

Bacteria were grown in 500 ml Erlenmeyer flasks (containing 100 ml of the growth medium) for 3–4 days at t = +32°C under intensive aeration in a Biorad orbital shaker ("Biorad Labs", Hungary). The bacterial growth was controlled on the ability to form individual colonies on the surface of solid (2% (w/w) agarose) media, as well as the optical density of the cell suspension measured on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) at λ = 540 nm in a quartz cuvette with an optical pathway length 10 mm.

Analytical determination of [^2H]inosine

Inosine was analytically determined in culture liquid samples with a volume of 10 μl on Silufol UV-254 chromatographic plates (150×150 mm) ("Kavalier", Czech Republic) using a standard set of ribonucleosides "Beckman-Spinco" (USA) in the solvent system: *n*-butanol–acetic acid–water (2:1:1, % (v/v)). Spots were eluted with 0.1 N HCl. The UV absorption of eluates was recorded on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) using a standard calibration plot. The level of bioconversion of the carbon substrate was assessed using glucose oxidase (EC 1.1.3.4).

Hydrolysis of intracellular polycarbohydrates

Dry deuterated biomass (50 mg) was placed into a 250 ml round bottomed flask, supplemented with 50 ml distilled ^2H_2O and 1.6 ml 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 on a T-24 centrifuge ("Heraues Separatech", Germany) (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 ("Beckman-Spinco", USA) sulfonated styrene (7.25% crosslinked) resin as a stationary phase; the granule diameter was 25 μm; 0.2 N sodium–citrate buffer (pH = 2.5) was used as an eluent; the working pressure – 50–60 atm; the eluent input rate – 18.5 ml/h; the ninhydrin input rate – 9.25 ml/h; detection at λ = 570 and λ = 440 nm (for proline).

Analysis of carbohydrates

Carbohydrates were analyzed on a Knauer Smartline chromatograph ("Knauer", Germany) equipped with a Gilson pump ("Gilson Inc.", Germany) and Waters K 401 refractometer ("Water Associates", Germany) using Ultrasorb CN C18 as a stationary phase: the column size – 250 × 10mm; the granule diameter – 10 μm; the mobile phase – acetonitrile–water (75 : 25, % (v/v); the input rate – 0.6 ml/min.

FAB mass spectrometry

FAB mass spectra were recorded on a VG-70 SEQ chromatograph ("Fisons VG Analytical", USA) equipped with a cesium source on a glycerol matrix with accelerating voltage 5 kV and ion current 0.6–0.8 mA.

EI mass spectrometry

EI mass spectra were recorded with an 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°C) after amino acid modification into methyl esters of N-5-dimethylamino(naphthalene)-1-sulfonyl (dansyl) amino acid derivatives according to an earlier elaborated protocol (Mosin & Ignatov, 2013).

IR spectroscopy

Samples of water for the research by the IR-spectroscopy method were taken from various sources of Bulgaria: 1 – hot mineral water (t = +75°C) from Rupite village (Bulgaria); 2 – sea water (Varna, Bulgaria); 3 – cactus juice of *Echinopsis pachanoi*. IR-spectra were registered by Dr. Kristina Chakarova (Bulgarian Academy of Sciences, Sofia, Bulgaria) on Fourier-IR spectrometer Brucker Vertex ("Brucker", Germany) (a spectral range: average IR – 370–7800 cm^{-1}; visible – 2500–8000 cm^{-1}; the permission – 0.5 cm^{-1}; accuracy of wave number – 0.1 cm^{-1} on 2000 cm^{-1}).

Results and discussion

Isotopic effects of deuterium in chemoheterotrophic bacterium *B. subtilis*

We have investigated isotopic effects of deuterium in prokaryotic cells of various taxonomic groups of microorganisms including chemoheterotrophic bacteria, which are believed to be at early stages of evolution, because glycolysis is accepted to be an ancient pathway of carbon assimilation. As a model for our experiments was used an inosine producer mutant strain of a Gram-positive aerobic spore-forming chemoheterotrophic bacterium *B. subtilis VKPM B-3157* [19] polyauxotrophic for histidine, tyrosine, adenine, and uracil (preliminary adapted to deuterium by selection of individual colonies on solid 2% (w/v) agarose growth media with 99.9 atom% ^2H_2O).

Most aerobic chemoheterotrophic bacteria are able to use as a source of growth substrates a variety of simple organic compounds (sugars, amino acids, organic acids). Some representatives of chemoheterotrophic bacteria can ferment carbohydrates. Some species do not require organic growth factors, while others require amino acids, growth factors, or vitamin B_2, B_6, B_{12} or both.

Most aerobic spore-forming bacteria are mesophylls with a temperature optimum between t = +30 °C and t = +45 °C, represented mainly by rod-shaped (Fig. 1).

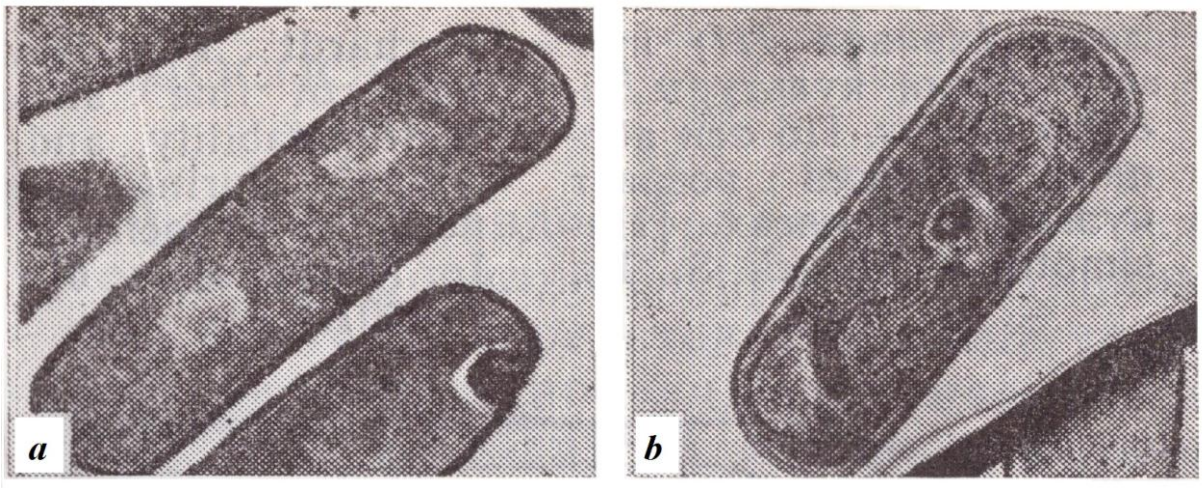


Figure 1: Electron micrographs of ultrathin sections of *B. subtilis*: *a*) – vegetative cells in the exponential growth phase; *b*) –combining of nucleoids with the formation of rod-shaped structures (adapted from Ryter A., 1965 [20])

Due to impaired metabolic pathways involved in the regulation of the biosynthesis of purine ribonucleosides (adenine, uracil, and inosine), this strain of a Gram-positive aerobic spore-forming chemoheterotrophic bacterium *B. subtilis VKPM B-3157* under standard growth conditions (PVC medium, late exponential growth, t = +32°C) synthesizes 17–20 gram of inosine per 1 liter of cultural medium [21]. The maximal inosine yield for this strain was reached on a protonated growth medium with 12% (w/v) glucose as a source of carbon and energy and 2% (w/v) yeast PVC as a source of growth factors and amine nitrogen. In our experiments it was necessary to replace the protonated growth substrates with their deuterated analogs, as well as to use ^2H_2O of high isotopic purity. For this purpose, we used autoclaved biomass of the Gram-negative facultative methylotrophic bacterium *Brevibacterium methylicum* B-5662 strain adapted to deuterium capable to assimilate methanol via the ribulose-5-monophosphate (RuMP) pathway of carbon assimilation. Owing to a 50–60% rate of methanol bioconversion (conversion efficiency, 15.5–17.3 gram of dry biomass per 1 gram of assimilated substrate) and stable growth in a deuterated M9 minimal medium with 98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol, this strain is the most convenient source for producing deuterated biomass; moreover, the cost of bioconversion is mainly determined by the cost of ^2H_2O and [^{2}H]methanol.

Table 1: Amino acid composition of hydrolyzed biomass of the facultative methylotrophic bacterium *B. methylicum* obtained on a 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*****
	Protonated sample (control)	**Sample from deuterated M9 medium**		
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	49.0
Isoleucine	4.14	3.64	5	49.0
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 were obtained for methyl esters of N-5-dimethylamino(naphthalene)-1-sulfonyl (dansyl) chloride amino acid derivatives.
** When calculating the level of deuterium enrichment, the protons(deuterons) at the carboxyl COOH- and amino 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.

The strategy for the biosynthesis of [^{2}H]inosine using biomass of *B. methylicum* as growth substrates was developed taking into account the ability of methylotrophic bacteria to synthesize large amounts of proteins (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 [22]. To provide high outputs of these compounds and minimize the isotopic exchange (^{1}H–^{2}H) in amino acid residues of protein molecules, the biomass was hydrolyzed by autoclaving in 0.5 *M* ^{2}HCl (in ^2H_2O). Since the *B. subtilis* inosine-producing strain is a polyauxotroph requiring tyrosine and histidine for its growth, we studied the qualitative and quantitative compositions of the amino acids in the hydrolyzed methylotrophic biomass produced in the maximally deuterated medium M9 (98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol), and the enrichment levels (Table 1). The methylotrophic hydrolysate contains 15 identified amino acids (except for proline, detectable at λ = 440 nm) with tyrosine and histidine contents per 1 gram of dry methylotrophic hydrolysate 1.82% and 3.72% (w/w), respectively, thereby surrisfying the auxotrophic requirements of the inosine producer strain of *B. subtilis* for these amino acids. The contents of other amino acids in the hydrolysate are also comparable with the needs of the strain in sources of carbon and amine nitrogen (Table 1). The indicator determining the high efficiency of deuterium incorporation into the synthesized product is high degrees of deuterium enrichment of amino acid molecules, which vary from 49 atom% ^{2}H for leucine/isoleucine to 97.5 atom% ^{2}H for alanine (Table 1). This allowed use the hydrolysate of deuterated *B. methylicum* biomass as a source of growth substrates for cultivating the *B. subtilis* inosine-producing strain.

The growth and biosynthetic characteristics of inosine-producing strain *B. subtilis* were studied on protonated yeast PVC medium with H_2O and 2% (w/w) yeast PVC and on an HW medium with 89% (v/v) ^2H_2O and 2% (w/w) hydrolysate of deuterated biomass of *B. methylicum* (Figure 1). The experiments demonstrated a certain correlation between the changes of growth dynamics of *B. subtilis* (Fig. 2, curves *1*, *1*'), output of inosine (Fig. 2, curves *2*, *2*'), and glucose assimilation (Fig. 3, 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 (Fig. 2, curve *2*). The output of inosine in the HW medium decreased 4.4-fold, reaching 3.9 g/l (Fig. 2, curve *2*'), and the level of glucose assimilation, 4-fold, as suggested by the remaining 40 g/l unassimilated glucose in cultural medium (Fig. 2, curve *3*'). The experimental data demonstrate that glucose is less efficiently assimilated during the growth in the HW medium as compared to the control conditions. This result demanded the examination of the content of glucose and other intracellular carbohydrates in the biomass of the inosine-producer strain of *B. subtilis*, which was performed by reverse phase HPLC on an Ultrasorb CN column (10 μm, 10×250 mm) with a mixture of acetonitrile–water (75:25, % (v/v)) as a mobile phase (Table 2). The fraction of intracellular carbohydrates in Table 2 (numbered according to the sequence of their elution from the column) comprises monosaccharides (glucose, fructose, rhamnose, and arabinose), disaccharides (maltose and sucrose), and four unidentified carbohydrates with retention times of 3.08 (15.63% (w/w)),

4.26 (7.46% (w/w)), 7.23 (11.72% (w/w)), and 9.14 (7.95% (w/w) min (not shown). As was expected, the output of glucose in the deuterated hydrolysate was 21.4% (w/w) of dry weight, that is, higher than the outputs of fructose (6.82% (w/w)), rhamnose (3.47% (w/w)), arabinose (3.69% (w/w)), and maltose (11.62% (w/w)) (Table 2). Their outputs in microbial biomass did not differ considerably related to the control in H_2O except for sucrose, which is undetectable in the deuterated sample. The levels of deuterium enrichment in carbohydrates varied from 90.7 atom% ^2H for arabinose to 80.6 atom% ^2H for glucose.

Table 2: Qualitative and quantitative compositions of intracellular carbohydrates isolated from *B. subtilis* after growing on HW-medium and levels of the deuterium enrichment*

Carbohydrate	Content in biomass, % (w/w) of 1 g of dry biomass		Level of deuterium enrichment of molecules, %**
	Protonated sample (control)	Sample from the HW medium	
Glucose	20.01	21.40	80.6±1.86
Fructose	6.12	6.82	85.5±1.92
Rhamnose	2.91	3.47	90.3±2.12
Arabinose	3.26	3.69	90.7±3.10
Maltose	15.30	11.62	–
Sucrose	8.62	ND**	–

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

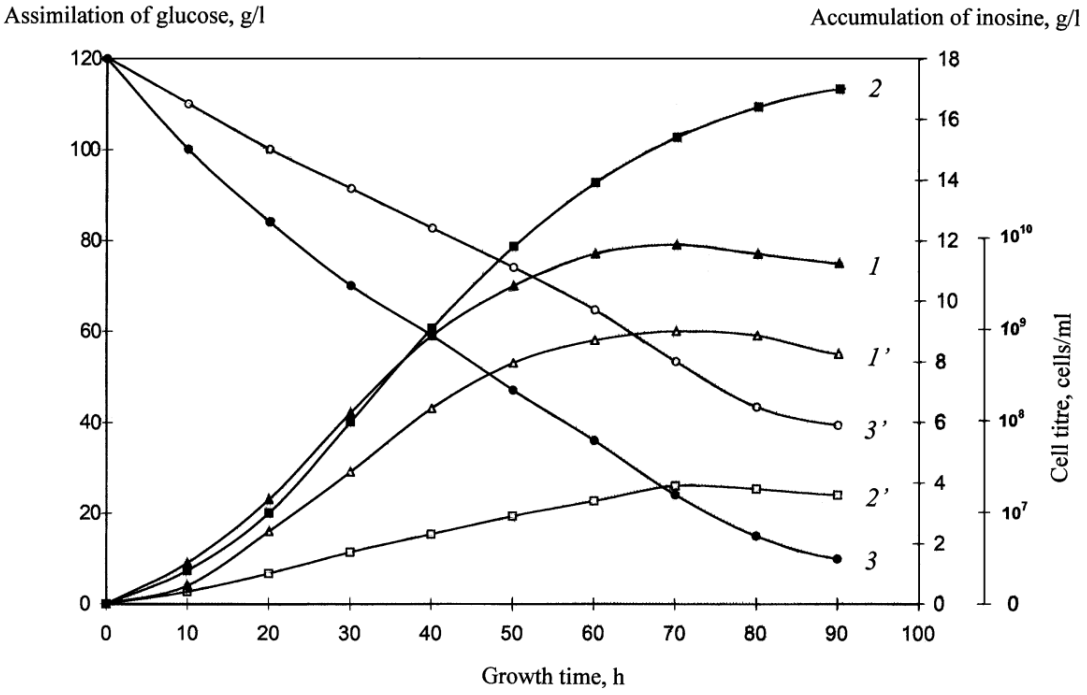


Figure 2. Growth dynamics of *B. subtilis* (*1*, *1*') (cells/ml), (*2*, *2*') inosine accumulation in cultural medium (g/l), and (*3, 3*') glucose assimilation (g/l) under different experimental conditions: (*1–3*) – a protonated yeast PVC medium and (*1*'–*3*') – HW medium with 2% (w/w) hydrolysate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

The using of a combination of physical-chemical methods for isolating [^{2}H]inosine from the cultural medium of the inosine producer strain was determined by the need for preparing inosine of a high chromatographic purity (no less than 95%). Since cultural medium contains inorganic salts, proteins, and polysaccharides, along with inosine, as well as accompanying secondary metabolites of nucleic nature (adenosine and guanosine) and unreacted substrates (glucose and amino acids), the cultural medium was fractionated in a stepwise manner for isolating [^{2}H]inosine. The fractionation 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 by low temperature precipitation with acetone at +4°C with subsequent adsorption of total ribonucleosides on activated carbon in the cold. The desorbed ribonucleosides were extracted from the reacted solid phase by eluting with EtOH–NH_3-solution at t = +60 °C; inosine – by extracting with 0.3 M ammonium–formate buffer (pH = 8.9) with subsequent crystallization in 80% (v/v) of 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. Figure 3 (curves *1–3*) shows UV-absorption spectra of inosine isolated from the cultural medium. The presence of major absorption band I, corresponding to natural inosine (λ_{max} = 249 nm, ε_{249} = 7100 M^{-1} cm^{-1}), as well as the absence of secondary metabolites II and III in the obtained sample (Fig. 2, curve *3*), demonstrates its uniformity and the efficiency of the isolation method.

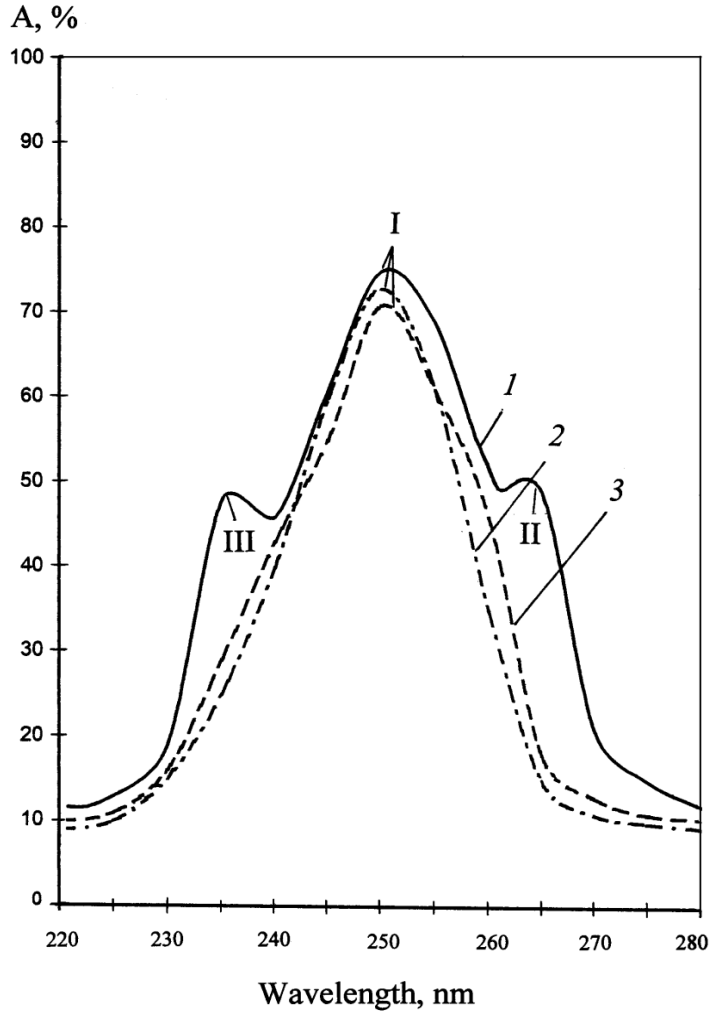


Figure 3: UV-absorption spectra of inosine (0.1 N HCl): (*1*) – initial LC after the growth of *B. subtilis* on HW medium; (*2*) – natural inosine, and (*3*) – inosine extracted from the LC. Natural inosine (*2*) was used as a control: (I) – inosine, (II, III) – secondary metabolites.

The level of deuterium enrichment of the [^{2}H]inosine molecule 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 4*b* regarding the control (natural protonated inosine, Figure 4*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 inosine molecule. Therefore, the molecular ion peak of inosine 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 4*a*). That result corresponds to five deuterium atoms incorporated into the inosine molecule (Figure 4b). 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 3).

Table 3: 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:

$$TLDE = \frac{[M]^+_{r1} \cdot C_2 + [M]^+_{r2} \cdot C_2 + ... + [M]^+_{rm} \cdot C_n}{\sum C_n}, \quad (1)$$

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

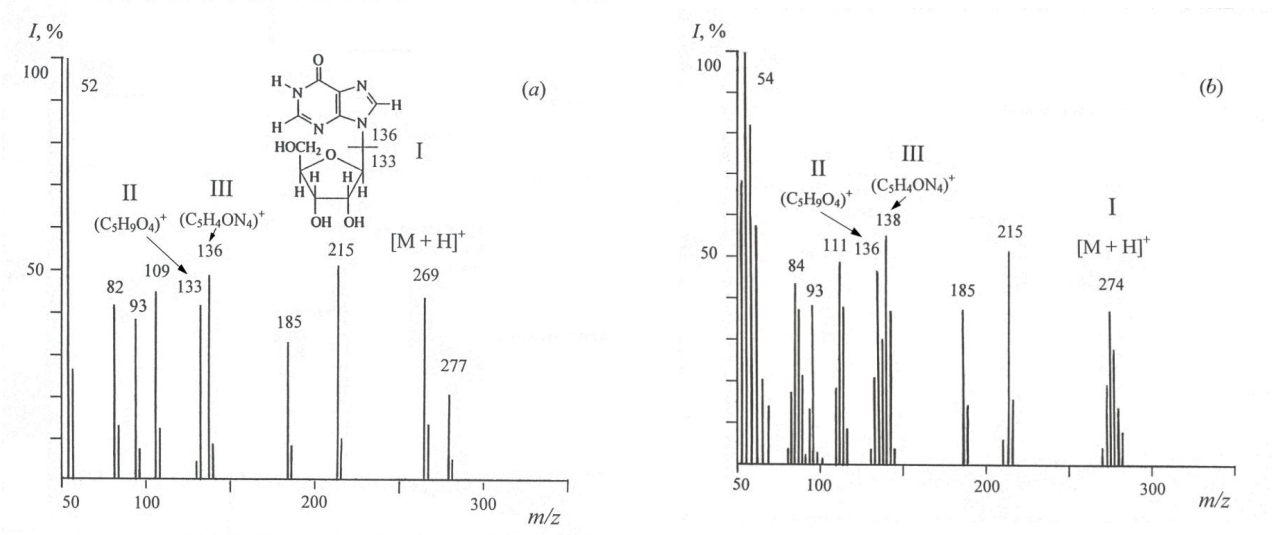


Figure 4: FAB mass spectra of inosine (glycerol as a matrix) under different experimental conditions: (*a*) – natural inosine; (*b*) – [^{2}H]inosine isolated from HW medium (scanning interval at *m/z* 50–350; major peaks with a relative intensity of 100% at *m/z* 52 and *m/z* 54; ionization conditions: 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 by the FAB-method, shown in Figure 5, 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 5). 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 *m/z* = 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 5). 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.

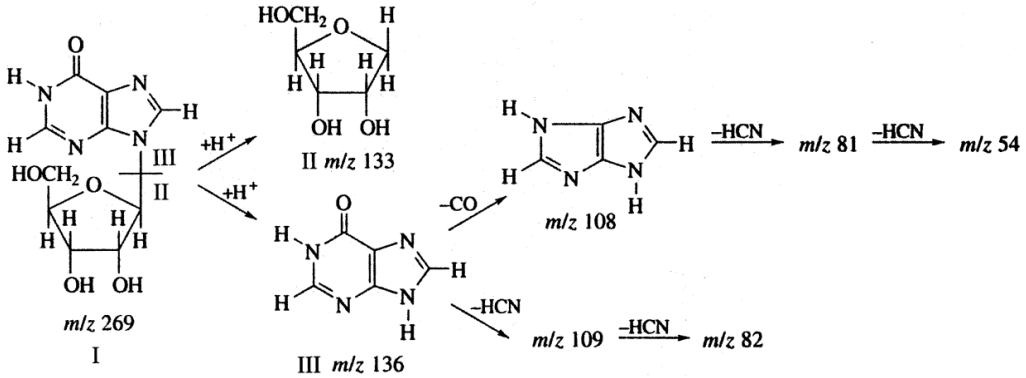


Figure 5: The fragmentation pathways of the inosine molecule leading to formation of smaller fragments by the FAB-method

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 (Fig. 6). 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 (C6), 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 (Fig. 6).

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:

$$3\ mol.\ glucose \rightarrow 2,3\text{-}butanediol + 2\ mol.\ glycerol + 4\ mol.\ CO_2$$

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:

$$2\ mol.\ glyceroldehyde\ 3\text{-}phosphate + 2\ mol.\ NAD^+ + 4\ mol.\ ADP + 2\ mol.\ P \rightarrow 2\ mol.\ pyruvate + 4\ mol.\ ATP + 2\ mol.\ NAD{\cdot}H + 2\ mol.\ H^+,$$

$$2\ mol.\ pyruvate + NAD{\cdot}H + H^+ \rightarrow 2\ mol.\ CO_2 + 2,3\text{-}butanediol + NAD^+$$

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

$$Glyceroldehyde\ 3\text{-}phosphate + NAD{\cdot}H + H^+ \rightarrow Glycerol + P + NAD^+$$

B. subtilis cannot grow under anaerobic conditions due to the use of glucose, probably because of the inability to recover glyceroldehyde 3-phosphate to glycerol [23]; in aerobic conditions, this bacterium ferments glucose to form large amounts of 2,3-butanediol.

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 enzymatic isomerization of glucose in ^2H_2O medium.

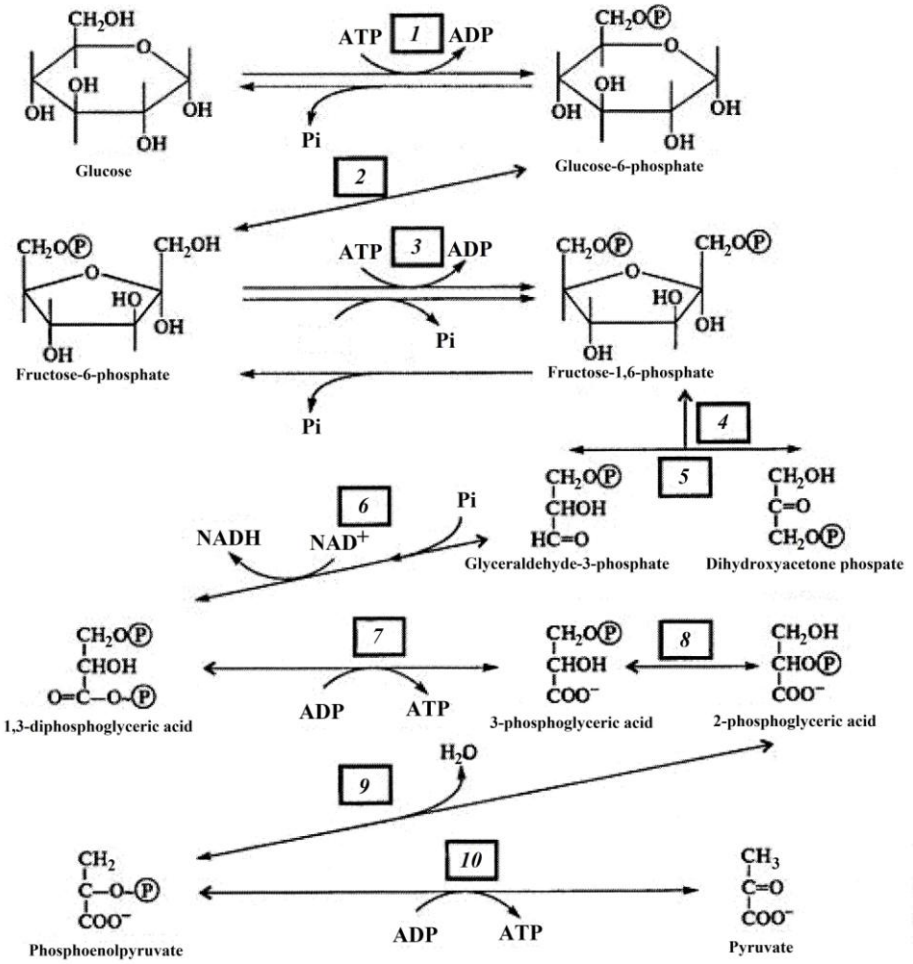


Figure 6: The Embden-Meyerhof pathway of glycolysis: 1 – hexokinase; 2 – glucose-6-phosphate izomerase; 3 – phosphofructokinase-1; 5 – fructose-1,6-bisphosphate aldolase; 6 – triosephosphate isomerase; 7 – glyceraldehyde-3-phosphat dehydrogenase; 8 – phosphoglycerate kinase; 9 – phosphoglycerate mutase; 10 – enolase; 11 – pyruvate kinase. Total reaction: Glucose + $2NAD^+ + 2ADP + 2P_i \rightarrow$ 2 piruvate + $2NADH + 2H^+ + 2ATP + 2H_2O$ (adapted from Stryer R.Y, 1995 [24])

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^-)- (C'2, C'3-positions in the ribose residue) and imidazole protons at NH^+ heteroatoms (N1-position in the hypoxanthine residue), 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 as glycine, glutamine and aspartate (with participation of $N^{10}–CHO–FH_4$ and $N^5,N^{10}–CH=FH_4$) (Fig. 7), that originated from the deuterated hydrolysate of methylotrophic bacterium *B. methylicum* obtained on 98 % of ^2H_2O medium. A glycoside proton at β-N_9-glycosidic bond could be replaced with deuterium *via* the reaction of CO_2 elimination at the stage of the ribulose-5-monophosphate formation from 3-keto-6-phosphogluconic acid with the subsequent proton (deuteron) attachment at the C1-position of ribulose-5-monophosphate. In general, our studies confirmed this scheme. However, it should be noted that auxotrophy of this mutant strain in tyrosine, histidine, adenine and uracil as well as the enzymatic synthesis of a precursor of inosine, inosine-5-monophospate (IMP) from ribose-5-monophosphate and amino acids

presupposes the branched metabolic pathways, different from those described above. Since it is known, that intermediates in glycolysis are precursors to a number of compounds as nucleotides and amino acids.

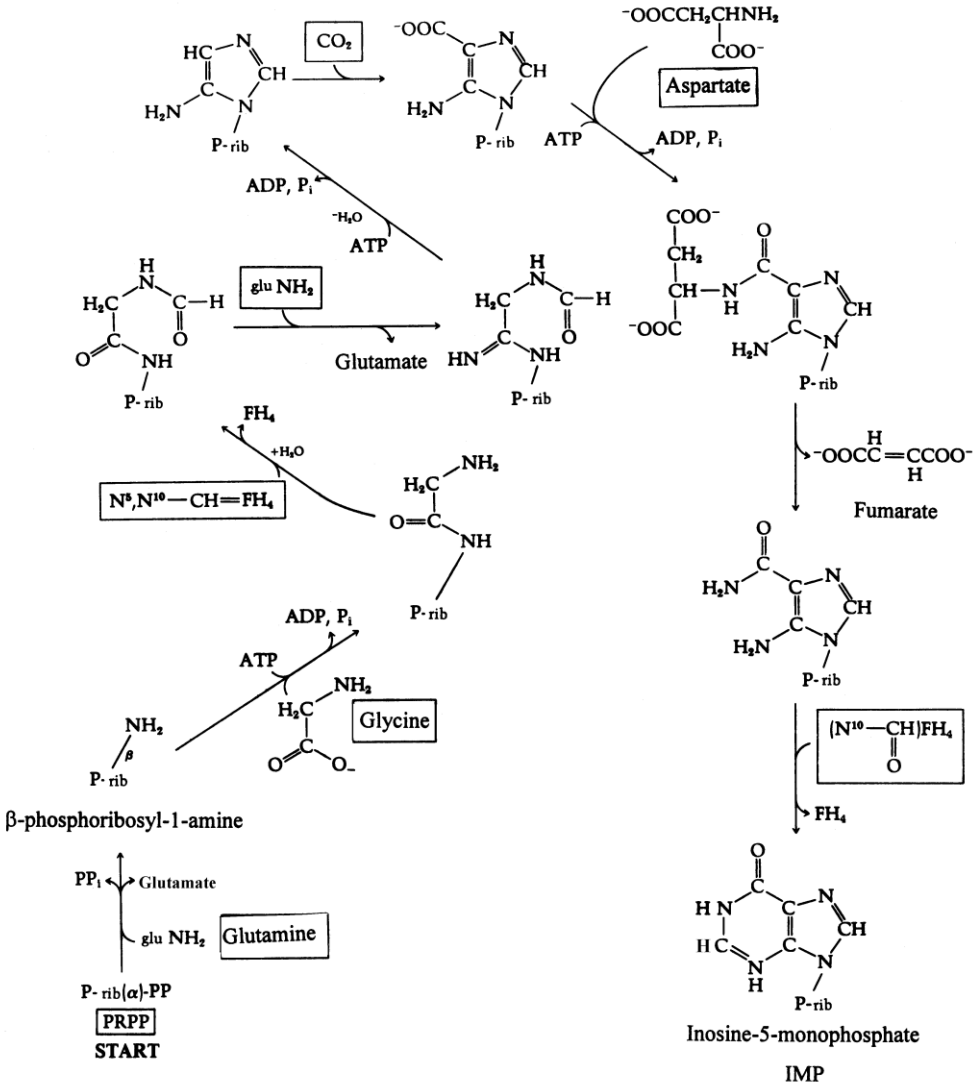


Figure 7: Overall scheme of biosynthesis of IMP by microbial cell (adapted from Bohinski, 1983 [25])

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–^2H can be synthesized de novo. Thus, the most sensitive to replacement of H 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 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.

The primary organisms (eobionts) were according to modern modern concepts heterotrophs, feeding by abiogenic organic substances [26]. In the process of life they emitted carbon dioxide, enriching the atmosphere. The atmosphere at that time was predominantly carbonic and did not contain oxygen. The first living organisms on Earth evidently originated in anaerobic conditions, when the primitive ocean was rich in organic matter formed at earlier stages of evolution [27]. The wide occurrence of glycolysis in bacteria indicates that it is one of the most ancient metabolic pathways [28]. The biochemical reactions of glycolysis and its parallel pathway, the pentose phosphate pathway, widespread in methylotrophs, occur metal-catalyzed under the oxygen-free conditions of the Archean ocean, and, probably, also in the absence of enzymes [29]. Glycolysis could thus have originated because of chemical limitations of the prebiotic world.

The main metabolic pathways as glycolysis and Krebs cycle are present in all living organisms and characterized to the universal common ancestor that was a prokaryote with combined amino acid, nucleotide, carbohydrate and lipid metabolism [30–32]. The preservation of these ancient pathways in evolution may result from the fact that these reactions are optimal for solving specific problems with metabolism. Thus, the end products of glycolysis and Krebs cycle are being formed with high efficiency and with a minimum number of steps. The first metabolic pathways based on enzymes might have been part of a purine nucleotide metabolism.

The oxygen-rich biosphere arose much later, about 2 billion years ago. This important geochemical revolution in evolution is explained by photosynthesis. With such a character of the evolution the aerobic chemo-autotrophs could appear only after oxygenic photosynthesis had been evolved. It can be assumed that chemo-autotrophs and chemo-heterotrophs could have evolved from common prokaryotic microorganisms precursors that carry out photosynthesis, but lost the photosynthesis apparatus, and their electron transport chain functioning in photosynthesis, began to carry out a new feature of assimilation of carbon compounds. Some contemporary representatives of the two major groups of prokaryotes, photosynthetic and non-photosynthetic ones, have very peculiar properties. These include the existence of several complex characteristic of these types of systems of internal membranes; absence of a functioning of tricarboxylic acid cycle; availability of the Calvin cycle, or its analogue, the pentose phosphate cycle; localization in carboxysomes the key enzyme of the Calvin cycle (ribulose diphosphate carboxylase) [33, 34]. Recent studies performed by us suggest the role of chemo-heterotrophs in the evolution of microorganisms [35]. Eukaryotic cells apparently arose only when there was oxygen in the atmosphere. All eukaryotes, with very few exceptions, are aerobic organisms. Prokaryotes occupy many different ecological niches. The development of various types of metabolism in prokaryotes was apparently due to a simple cell structure, highly regulation systems, a rapid growth and the presence of multiple gene transfer mechanisms. On the path of further evolution of prokaryotes there were insurmountable difficulties related primarily to the small size of the genome, its haploid state and the small size of the cells. The new environment with aerobic conditions allows to obtain more energy, but to its use it was needed larger cells, extensive structural differentiation and therefore on many times greater gene. Large and small biological molecules provide the biosynthesis, metabolism and bioenergetics. The wastes of primary protozoa were compounds such as lactic acid and ethanol. These compounds had much less energy consumption compared to carbohydrates, but they were able to release a large amount of energy if fully oxidized to CO_2 and H_2O. As a result of the evolution originated new living organisms capable to fix oxygen in the form of H_2O and CO_2, and in return to receive the energy of combustion of what was formerly their waste.

Metabolic processes that occur with the participation of oxygen (primarily oxidative phosphorylation in breathing), and relatively few are evolutionarily later than anaerobic processes. In the absence of oxygen, it is impossible to complete combustion (oxidation) of the organic molecules of nutrients. However, as was demonstrated by the properties of the currently existing anaerobic cells, the essential for life energy is being obtained in the course of redox processes. In aerobic systems the final acceptor (oxidizing agent) of hydrogen serves oxygen, while in anaerobic – other substances. Oxidation without oxygen is implemented in two fermentation

pathways – glycolysis and alcoholic fermentation. Glycolysis consists in splitting of multistage hexoses up to two molecules of pyruvate (pyruvic acid) containing three carbon atoms. In this way the two molecules of NAD reduced to NADH and two molecules of ADP phosphorylated to get two molecules of ATP.

The comparison of existing metabolic pathways of amino acids with the genetic code reveals that a metabolically related amino acids correlate well with respect to their codon. This makes it a very attractive idea of parallel evolution of the genetic code and metabolism and indicates the presence of the hierarchy of amino acids. More simple amino acids such as Gly, Ser, Ala, Asp and Glu are considered earlier in contrast to the more complex amino acids, as Met, His and Asn. However, consistent appearance of amino acids does not reflected in the existing protein structures, since amino acid residues of proteins to some extent are interchangeable, so the correlation with early life periods is currently hardly justified [36].

Possible conditions for origin of first organic forms in hot mineral water with HDO

Biological experiments with ^2H_2O allow to better prognosticate the conditions under which life and living matter had evolved [37]. The mineral composition of the water (Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, K^+, Na^+, SO_4^{2-}, Cl^-), the isotopic composition, the pH value and temperature appear to play a significant role in evolution of first organic forms [38]. Most better to these conditions satisfy karst water and seawater. Circulating in bowels of cracks, crevices, channels and caves karst waters are enriched with $Ca(HCO_3)_2$ and other minerals, actively cooperating with living matter. Once appeared in these waters the process of self-organization of primary organic forms in water solutions might be supported by thermal energy of magma, volcanic activity and solar radiation.

We have conducted experiments for the testing of various samples of mineral water from karst springs and sea water from Bulgaria and the cactus juice of *Echinopsis pachanoi* with IR-spectroscopy and Differential Non-equilibrium Energy Spectrum (DNES) method relative to the control – deionized water. The cactus is chosen as a model system because it contains approximately 90% (w/w) H_2O (Table 4).

Table 4: Characteristics of spectra of water of various origin obtained by the DNES-method*

-E, eV			λ, μm	k, cm^{-1}
Cactus juice	**Mineral water from Rupite Village (Bulgaria)**	**Seawater**		
0.1112	0.1112	–	11.15	897
0.1187	0.1187	–	10.45	957
0.1262	0.1262	–	9.83	1017
0.1287	0.1287	–	9.64	1037
0.1362	–	0,1362	9.10	1099
0.1387	0.1387	–	8.95	1117

Notes:

*The function of the distribution of energies Δf among individual H_2O molecules was measured in reciprocal electron volts (eV^{-1}). It is shown at which values of the spectrum -E (eV) are observed the biggest local maximums of this function; λ – wave length; κ – wave number.

For calculation of the function *f(E)* which represents the energy spectrum of water, the experimental dependence between the wetting angle (θ) and the energy of hydrogen bonds (*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:

$$\theta = arcos\ (-1 - 14.33E) \qquad (2)$$

According to the experimental data the closest to the DNES-spectrum of cactus juice (Fig. 8, *curve 1*) was the DNES-spectrum of mineral water contacting Ca^{2+} and HCO_3^- ions (Fig. 8, *curve 2*). DNES-spectra of cactus juice and mineral water have magnitudes of local maximums at E = –0.1112; -0.1187; -0.1262; -0.1287 and –0.1387 eV. Similar local maximums in the DNES-spectrum between cactus juice and seawater were detected at E = -0.1362 eV. The DNES-spectrum of the control sample of deionized water (Fig. 8, *curve 5*) was substantially different from the spectra of seawater and mineral water.

Another important parameter was measured by the DNES method – the average energy ($\Delta E_{H...O}$) of hydrogen H...O-bonds among individual molecules H_2O, which makes up -0,1067±0,0011 eV. When the water temperature is changed, the average energy of hydrogen H...O-bonds alternates. This testified about the restructuring of average energies among individual H_2O molecules with a statistically reliable increase of local maximums in DNES-spectra.

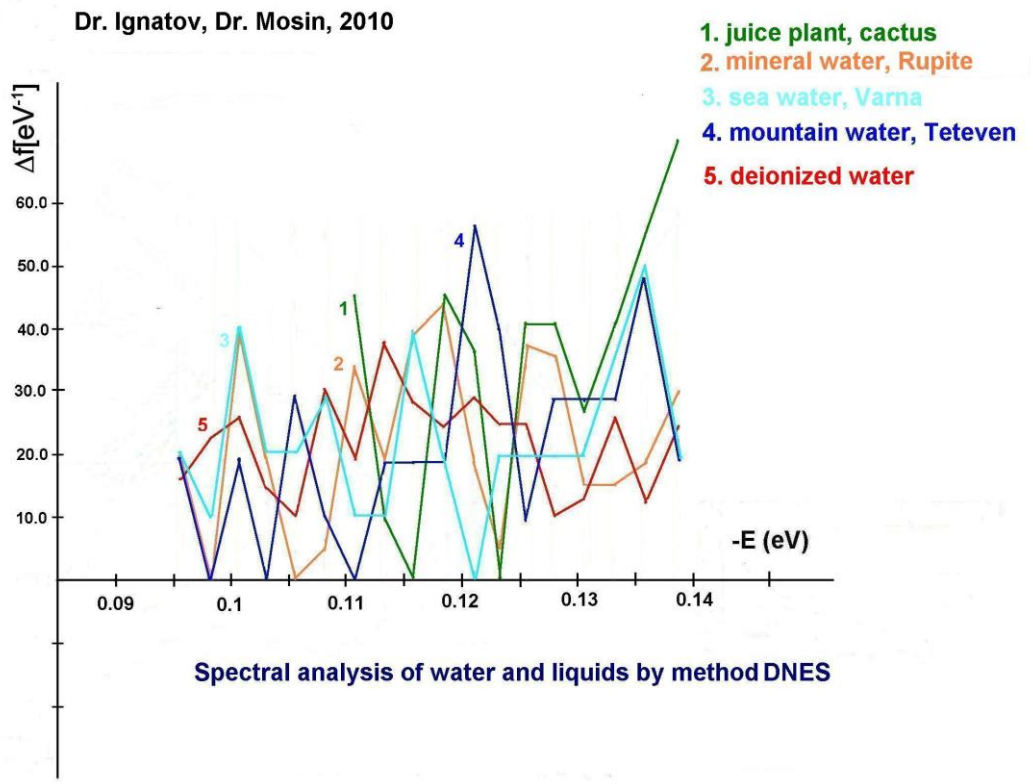


Figure 8: DNES-spectra of water samples of various origin: 1 – cactus juice; 2 – mineral water from Rupite village (Bulgaria); 3 – seawater (Varna, Bulgaria); 4 – mountain water (Teteven, Bulgaria); 5 – deionized water (the control)

As was shown from these data, the closest to the IR-spectrum of cactus juice was mineral water from Rupite Village (Bulgaria), which DNES and IR spectrum is shown in Fig. 8 and Fig. 9 (Thermo Nicolet Avatar 360 Fourier-transform IR). IR-spectra of cactus juice and mineral water with HCO_3^- (1320–1488 mg/l), Ca^{2+} (29–36 mg/l), pH (6.85–7.19), have local maximums at λ = 8.95; 9.67; 9.81; 10.47 and 11.12 μm (Fourier-IR spectrometer Brucker Vertex). Common local maximums in the IR-spectrum between cactus juice and seawater are detected at λ = 9.10 μm.

The local maximums obtained with the IR method at λ = 9.81 μm (k = 1019 cm^{-1}) and λ = 8.95 μm (k = 1117 cm^{-1}) (Thermo Nicolet Avatar 360 Fourier-transform IR) are located on the spectral curve of the local maximum at λ = 9.7 μm (k = 1031 cm^{-1}) (Fig. 9). With the DNES method were obtained the following results – 8.95; 9.10; 9.64; 9.83; 10.45 and 11.15 μm (λ, wavelength), or 897; 957; 1017; 1037; 1099 and 1117 cm^{-1} (k, wave numbers).

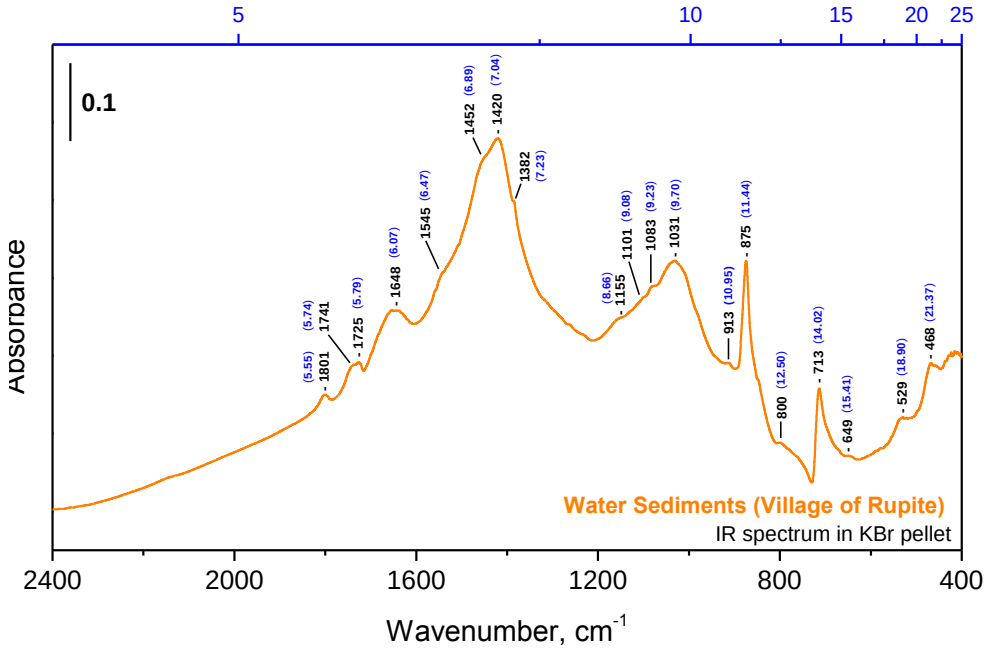


Figure 9: IR-spectrum of water obtained from Rupite Village (Bulgaria)

Such a character of IR- and DNES-spectra and distribution of local maximums may prove that hot mineral alkaline water having the most likeness in the characters of DNES- and IR-spectra and its characteristics with cactus juice is preferable for origin and maintenance of life compared to other types of water analyzed by these methods. Thus, in hot mineral waters the local maximums in the IR-spectrum are more manifested compared to the local maximums obtained in the IR-spectrum of the same water at a lower temperature. The difference in the local maximums from +20 °C to +95 °C at each +5 °C according to the Student t-criterion makes up – $p < 0.05$. These data indicate that the origination of life and living matter depends on the structure and physical chemical properties of water, as well as its temperature and pH value. The most closed to the IR- and DNES-spectrum of water, which contains bicarbonates and Ca^{2+} ions typical for the formation of stromatolites, the dolomite layered acretionary structures formed in shallow seawater by colonies of cyanobacteria, is the IR-spectrum of cactus juice. For this reason cactus juice was applied as a model system. The most closed to local maximums in IR-spectrum of cactus juice are local maximums in IR-spectra of alkaline mineral water interacting with $CaCO_3$ and then seawater. In connection with these data the following reactions participating with $CaCO_3$ in aqueous solutions are important:

$$CO_2 + 4H_2S + O_2 = CH_2O + 4S + 3H_2O, \quad (3)$$
$$CaCO_3 + H_2O + CO_2 = Ca(HCO_3)_2, \quad (4)$$
$$CO_2 + OH^- = HCO_3^- \quad (5)$$
$$2HCO_3^- + Ca^{2+} = CaCO_3 + CO_2 + H_2O \quad (6)$$

The equation (3) shows how some chemosynthetic bacteria use energy from the oxidation of H_2S and CO_2 to S and formaldehyde (CH_2O). The equation (4) is related to one of the most common processes in nature: in the presence of H_2O and CO_2, $CaCO_3$ transforms into $Ca(HCO_3)_2$. In the presence of hydroxyl OH^- ions, CO_2 transforms into HCO_3^- (equation (5). Equation (6) is valid for the process of formation of the stromatolites – the dolomite layered acretionary structures formed in shallow seawater by colonies of cyanobacteria. In 2010 D. Ward described fossilized

stromatolites in the Glacier National Park (USA) [39]. Stromatolites aged 3.5 billion years had lived in warm and hot water in zones of volcanic activity, which could be heated by magma [40]. This suggests that the first living forms evidently evolved in hot geysers [41]. It is known that water in geysers is rich in carbonates, while the temperature is ranged from +100 °C to +150 °C. In 2011 a team of Japanese scientists under the leadership of T. Sugawara showed that life originated in warm or, more likely, hot water [42]. From aqueous solution of organic molecules, DNA and synthetic enzymes were created proto cells. For this the initial solution was heated to a temperature close to water's boiling point +95 °C. Then its temperature was lowered to +65 °C with formation of proto cells with primitive membrane. This laboratory experiment is an excellent confirmation of the possibility that life originated in hot water.

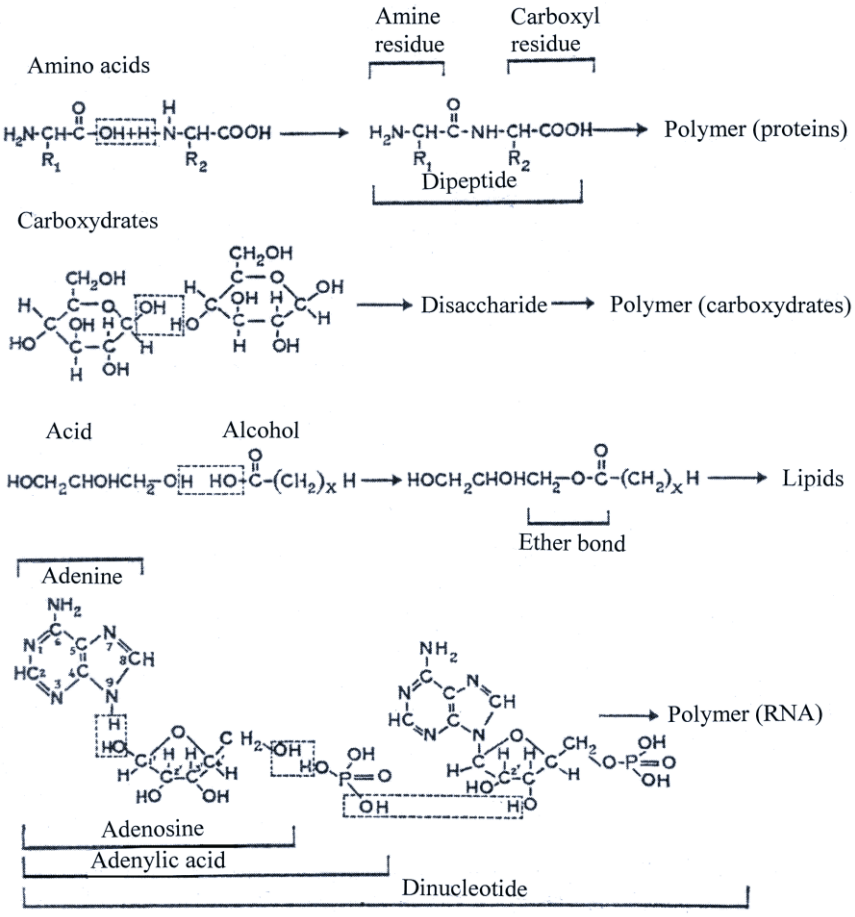


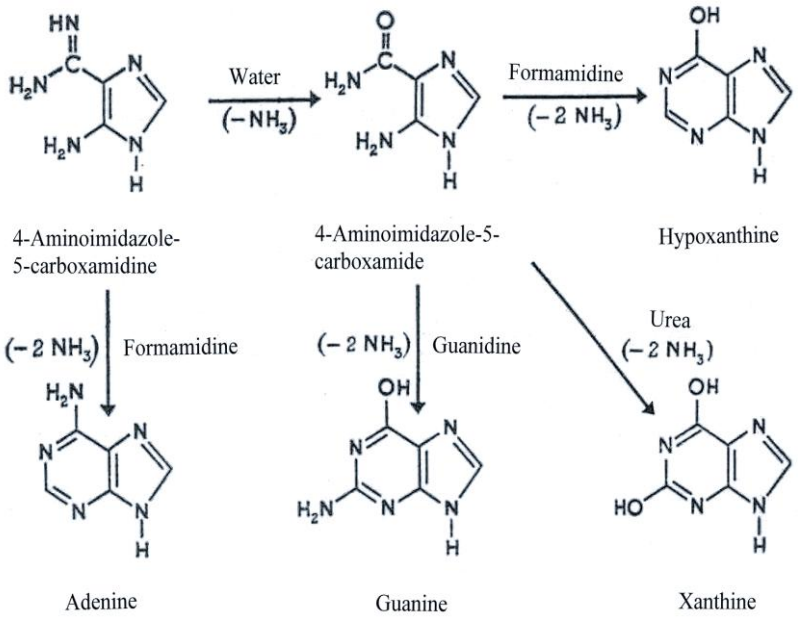
Figure 10: Reactions of condensation and dehydration in alkaline conditions with pH = 9–10 catalyzed by HCN and its derivatives, resulting in synthesis from separate molecules larger organic molecules of polymers. The top three equations: condensation and the subsequent polymerization of amino acids in proteins; carbohydrates – in polycarboxydrates and acids and ethers – into lipids. The bottom equation – condensation of adenine with ribose and H_3PO_4, leading to formation of dinucleotide

Analyzing the experimental data the prognosis was made to predict a possible transition from synthesis of small organic molecules under high temperatures to more complex organic molecules as proteins. There are reactions of condensation-dehydration of amino acids into separate blocks of peptides that occur under alkaline conditions, with pH = 9–11. The important factor in reaction of condensation of two amino acid molecules into the dipeptide is allocation of H_2O molecule when a peptide chain is formed, as the reaction of polycondensation of amino acids is accompanied by dehydration, the H_2O removal from reaction mixture speeds up the reaction rates. This testifies that formation of early organic forms may have occured nearby active volcanoes, because at early periods of geological history volcanic activity occurred more actively than during subsequent

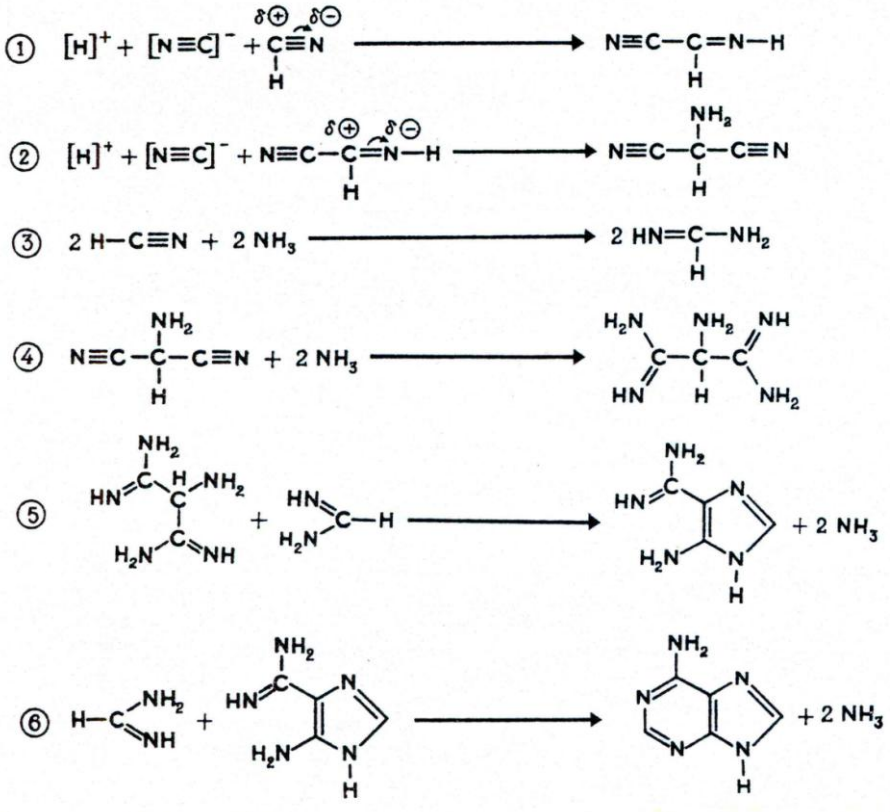
geological times. However, dehydratation accompanies not only amino acid polymerization, but also association of other small blocks into larger organic molecules, and also polymerization of nucleotides into nucleic acids. Such association is connected with the reaction of condensation, at which from one block a proton is removed, and from another – a hydroxyl group with the formation of H_2O molecule.

In 1969 the possibility of existence of condensation-dehydration reactions under conditions of primary hydrosphere was proven by M. Calvin [43]. From most chemical substances hydrocyanic acid (HCN) and its derivatives – cyanoamid (CH_2N_2) and dicyanoamid ($HN(CN)_2$) possess dehydration ability and the ability to catalyze the process of linkage of H_2O from primary hydrosphere [44]. The presence of HCN in primary hydrosphere was proven by S. Miller's early experiments [45]. Chemical reactions with HCN and its derivatives are complex with a chemical point of view; in the presence of HCN, CH_2N_2 and $HN(CN)_2$ the condensation of separate blocks of amino acids accompanied by dehydration, can proceed at normal temperatures in strongly diluted H_2O-solutions. These reactions show the results of synthesis from separate smaller molecules to larger organic molecules of polymers, e.g. proteins, polycarboxydrates, lipids, and nucleic acids (Fig. 10). Furthermore, polycondensation reactions catalyzed by HCN and its derivatives depend on acidity of water solutions in which they proceed [46]. In acid aqueous solutions with pH = 4–6 these reactions do not occur, whereas alkaline conditions with pH = 9–10 promote their course. There has not been unequivocal opinion, whether primary water was alkaline, but it is probable that such pH value possessed mineral waters adjoining with basalts, i.e. these reactions could occur at the contact of water with basalt rocks, that testifies our hypothesis. It may be supposed that primary water might contain more deuterium at early stages of life evolution, and deuterium was distributed non-uniformly in hydrosphere and atmosphere [47]. The reason of this is that the primary atmosphere of the Earth was reductive, without O_2–O_3 layer protecting the Earth surface from rigid short-wave solar radiation carrying huge energy. This simplifies radiation to freely pass through O_2-free atmosphere and reaching hydrosphere, may be the cause of further radiolysis and photolysis of water. Energy of radiation, volcanic geothermal processes on a hot Earth surface and electric discharges in atmosphere, could lead to the accumulation of deuterium in hydrosphere in the form of H^2HO that evaporates more slowly that H_2O, but condenses faster. This fact may make inprint on thermostability of deuterated macromolecules as the covalent bonds formed with ^2H are stronger than those formed with hydrogen.

It should be noted, that geothermal sources might be used for synthesis of various organic molecules. Thus, amino acids were detected in solutions of formaldehyde CH_2O with hydroxylamine NH_2OH, formaldehyde with hydrazine (N_2H_4) in water solutions with HCN, after heating of a reactionary mixture to +95 ^{0}C [48]. In model experiments reaction products were polymerized into peptide chains, that is the important stage towards inorganic synthesis of protein. In a reactionary mixture with a HCN–NH_3 solution in water were formed purines and pyrimidines (Fig. 11). In other experiments amino acid mixtures were subjected to influence of temperatures from +60 ^{0}C up to +170 ^{0}C with formation of short protein-like molecules resembling early evolutionary forms of proteins subsequently designated as thermal proteinoids. They consisted of 18 amino acids usually occurring in protein hydrolyzates. The synthesized proteinoids are similar to natural proteins on a number of other important properties, e. g. on linkage by nucleobases and ability to cause the reactions similar to those catalyzed by enzymes in living organisms as decarboxylation, amination, deamination, and oxidoreduction. Proteinoids are capable to catalytically decompose glucose [49] to have an effect similar to the action of α-melanocyte-stimulating hormone [50]. The best results on polycondensation were achieved with the mixes of amino acids containing aspartic and glutamic acids, which are essential amino acids occurring in all modern living organisms. Under certain conditions in hot mixture of proteinoids in water solutions are formed elementary structures like proteinoid microspheres with diameter 5–10 μm [51].



a)



b)

Figure 11: Prospective mechanisms of thermal (+95 °C) synthesis of purines in aqueous solutions: *a)* – synthesis of hypoxanthine, adenine, guanine and xanthine from 4-aminoimidazole-5-carboxamidine, 4-aminoimidazole-5-carboxamide, water, NH_3, formamidine and urea; *b)* – synthesis of adenine from NH_3 and HCN (total reaction: 5HCN = adenine)

Taking into account the resent experimental data it may be concluded that the initial stage of evolution, apparently, was connected with the formation at high temperature the mixtures of amino acids and nitrogenous substances – analogues of nucleic acids. Such synthesis is possible in aqueous solutions under thermal conditions in the presence of H_3PO_4. The next stage is the polycondensation of amino acids into thermal proteinoids at temperatures +65...+95 °C. After that stage in a mix of thermal proteinoids in hot water solutions were formed the membrane like structures. The first living structures were most probably formed in warm and hot mineral water with more bicarbonate and metal ions (Na, Ca, Mg, Zn, K, etc.). The role of metal ions in metabolism consists in their using as catalysts of various metabolic reactions, while the metal complexes with organic molecules activate the organic molecules and organize the biochemical reactions. By activating it should be understood the polarization (stabilization of negative charges), and organization means the strict spatial arrangement of molecules or ions involved in the reaction. Therefore, the most important function of the metal complexes consists in strengthening the role of the systems of spatial codes toward the ways of chemical evolution.

Conclusion

The experimental data indicate that origination of life and living matter depends on physical-chemical properties of water and external factors – temperatures, pH, electric discharges and isotopic composition. Hot mineral alkaline water interacting with $CaCO_3$ is most closed to these conditions. Next in line with regard to its quality is seawater. For chemical reactions of dehydration-condensation to occur in hot mineral water, water is required to be alkaline with pH range 9–11. In warm and hot mineral waters the local maximums in IR-spectra from 8 to 14 μm were more expressed in comparison with the local maximums measured in the same water samples with lower temperature. The content of deuterium in hot mineral water may be increased due to the physical chemical processes of the deuterium accumulation as solar radiation, volcanic geothermal processes and electric discharges in the atmosphere. These natural processes could lead to the enrichment of the hydrosphere by deuterium in the form of HDO which evaporates more slowly than H_2O, and condenses faster. We had a perspective selection of chemoheterotrophic bacteria for our research as they are the microorganisms located on the lower stage of evolutionary development, and quickly adapt to changing environmental factors. The taxonomy of a Gram-positive chemoheterotrophic bacterium *Bacillus subtilis* and its resistance to deuterium was also analyzed on an evolutionary level taking into account the hydrological conditions of primodial hydrosphere and the presence of HDO, as well as the qualitative and quantitative composition of the cellular protein, amino acids and carbohydrates on media with maximum deuterium content. It was demonstrated on the example of chemoheterotrophic bacteria that first heterotrophic microorganisms might have been originated in hot mineral water at t = + 65–95 °C and pH = 9–11 that is more suitable for maintenance and origin of life than other analyzed water samples.

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Studying of Biosynthetic Pathways of ^{2}H-labeled Purine Ribonucleoside Inosine in a Chemoheterotrophic Bacterium *Bacillus subtilis B-3157* by FAB Mass-Spectrometry

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Abstract

This paper deals with studying biosynthetic pathways of ^{2}H-labeled purine ribonucleoside inosine excreted into liquid microbial culture (LC) by Gram-positive chemoheterotrophic bacterium *Bacillus subtilis B-3157* while growing of this bacterium on heavy water (HW) medium with 2% (v/v) hydrolysate of deuterated biomass of the methylotrophic bacterium *Brevibacterium methylicum B-5662* as a source of ^{2}H-labeled growth substrates. Isolation of ^{2}H-labeled inosine from LC was performed by adsorption/desorption on activated carbon with following extraction by 0,3 M ammonium–formate buffer (pH = 8,9), crystallization in 80% (v/v) EtOH, and ion exchange chromatography (IEC) on a column with AG50WX 4 cation exchange resin equilibrated with 0,3 M ammonium–formate buffer and 0,045 M NH_4Cl. The investigation of deuterium incorporation into the inosine molecule by FAB method demonstrated incorporation of 5 deuterium atoms into the molecule (the total level of deuterium enrichment – 65,5 atom% ^{2}H) with 3 deuterium atoms being included into the ribose and 2 deuterium atoms – into the hypoxanthine residue of the molecule. Three non-exchangeable deuterium atoms were incorporated into the ribose residue owing to the preservation in this bacterium the minor pathways of *de novo* glucose biosynthesis in ^2H_2O-medium. These non-exchangeable deuterium atoms in the ribose residue were originated from HMP shunt reactions, while two other deuterium atoms at C2, C8-positions in the hypoxanthine residue were synthesized from [^{2}H]amino acids, primarily glutamine and glycine, that originated from deuterated hydrolysate. A glycoside proton at β-N_9-glycosidic bond could be replaced with deuterium *via* the reaction of CO_2 elimination at the stage of ribulose-5-monophosphate formation from 3-keto-6-phosphogluconic acid with subsequent proton (deuteron) attachment at the C1-position of ribulose-5-monophosphate. Two other protons at C2(C3) and C4 positions in ribose residue could be replaced with deuterium *via* further enzimatic isomerization of ribulose-5-monophosphate into ribose-5-monophosphate.

Keywords: ^{2}H-labeled inosine, biosynthesis, metabolism, heavy water, *Bacillus subtilis*, FAB-mass-spectrometry.

Introduction

Natural nucleosides labeled with deuterium (^{2}H) are of considerable scientific and practical interest for various biochemical and diagnostic purposes [1], structure-function studies [2], and research into cell metabolism [3]. Their usage is determined by the absence of radiation danger and the possibility of localizing the deuterium label in a molecule by ^{1}H-NMR [4], IR spectroscopy [5] and mass spectrometry [6] methods. The latter seems more preferable due to high sensitivity of the method and possibility to study the distribution of deuterium label *de novo*. The recent advance in technical and computing capabilities of these analytical methods has allowed a considerable increase the efficiency of carrying out biological studies with ^{2}H-labeled molecules *de novo*, as well as to carry out the analysis of the structure and function of nucleosides and their analogs at the molecular level [7]. In particular, ^{2}H-labeled ribonucleosides and their analogs are used in template-directed syntheses of deuterated RNA macromolecules for studying their spatial structure and conformational changes [8]. Perdeuteration and selective deuteration techniquemay be useful approaches for simplification of NMR spectra and for other structural studies of large biomolecules. Driven by the progress in multinuclear multidimensional NMR spectroscopy, deuteration of nucleic acids has especially found wide applications in the NMR studies of these macromolecules in solution. Deuterated ribonucleosides may be of further interest for NMR spectroscopy studies. Another usage of these deuterated molecules has been in atom transfer and kinetic isotope effect experiments.

An important factor in studies with ^{2}H-labeled nucleosides and their analogs is their availability. ^{2}H-labeled nucleosides can be synthesized with using chemical, enzymatic, and microbiological methods [9, 10]. Chemical synthesis is frequently multistage; requires expensive reagents and ^{2}H-labeled substrates, and eventually results to a racemic mixture of D- and L-enantiomers, requiring special methods for their separation [11]. Finer chemical synthesis of [^{2}H]nucleosides combine both chemical and enzymatic approaches [12].

Microbiology proposes an alternative method for synthesis of [^{2}H]nucleosides, applicable for various scientific and applied purposes; the main characteristics of the method are high outputs of final products, efficient deuterium incorporation into synthesized molecules, and preservation of the natural L-configuration of ^{2}H-labeled molecules [13]. A traditional approach for biosynthesis of ^{2}H-labeled natural compounds consists in growing of strains-producers on growth media containing maximal concentrations of $^{2}H_2O$ and ^{2}H-labeled substrates [14]. However, the main obstacle seriously implementing this method is a deficiency in ^{2}H-labeled growth substrates with high deuterium content. First and foremost, this stems from a limited availability and high costs of highly purified deuterium itself, isolated from natural sources. The natural abundance of deuterium makes up 0,0015 atom%; however, despite a low deuterium content in specimens, recently developed methods for its enrichment and purification allow to produce ^{2}H-labeled substrates with high isotopic purity.

Starting from first experiments on the growth of biological objects in heavy water, the approach involving hydrolysates of deuterated bacterial and micro algal biomass as growth substrates for growth of other bacterial strains-producers have been developed in this country [15]. However, these experiments discovered a bacteriostatic effect of $^{2}H_2O$ consisted in inhibition of vitally important cell functions in $^{2}H_2O$; this effect on micro algal cells is caused by 70% (v/v) $^{2}H_2O$ and on protozoan and bacterial cells – 80–90% (v/v) $^{2}H_2O$ [16]. Attempts to use biological organisms of various taxonomic species, including bacteria, micro algae, and yeasts [17] for growth in $^{2}H_2O$ have not been widely used because of complexity of biosynthesis, consisted in need of complex growth media, applying intricate technological schemes, etc. That is why a number of applied items regarding the biosynthesis of natural ^{2}H-labeled compounds in $^{2}H_2O$ remains to be unstudied.

More promising seem the technological schemes involving as a source of ^{2}H-labeled growth substrates the biomass of methylotrophic bacteria, assimilating methanol *via* the ribulose-5-monophosphate (RMP) and serine pathways of carbon assimilation [18]. The assimilation rate of methylotrophic biomass by prokaryotic and eukaryotic cells makes up 85–98% (w/w), and their productivity calculated on the level of methanol bioconversion into cell components reaches 50–

60% (w/w) [19]. As we have earlier reported, methylotrophic bacteria are convenient objects able to grow on minimal salt media containing 2–4% (v/v) [^{2}H]methanol, whereon other bacteria are unable to reproduce, and may easily be adapted to maximal ^2H_2O concentrations, that is the most important for the biosynthesis of ^{2}H-labeled natural compounds [20].

The aim of this research was studying the biosynthetic pathwaysof ^{2}H-labeled inosine usung a Gram-positive chemoheterotrophic bacterium *Bacillus subtilis B-3157* by FAB-method.

Material and methods

Bacterial strain

The object of the research was a strain of inosine producer, spore-forming aerobic Gram-positive chemoheterotrophic bacterium *B. subtilis B-3157*, polyauxotrophic for histidine, tyrosine, adenine, and uracil (demand, 10 mg/l), obtained from Institute of Genetics and Selection of Industrial Microorganisms (Russia). The initial strain was adapted to deuterium by plating individual colonies onto 2% (w/v) agarose with stepwise increasing gradient of ^2H_2O concentration and subsequent selection of individual cell colonies stable to the action of ^2H_2O.

Chemicals

Growth media were prepared using ^2H_2O (99,9 atom% ^{2}H), ^{2}HCl (95,5 atom% ^{2}H), and [^{2}H]methanol (97,5 atom% ^{2}H), purchased from JSC "Izotop" (St. Petersburg, Russia). Inorganic salts, D- and L-glucose ("Reanal", Hungary) were preliminary crystallized in ^2H_2O. ^2H_2O was distilled over $KMnO_4$ with subsequent control of the isotope purity by ^{1}H-NMR spectroscopy on a Brucker WM-250 ("Brucker Daltonics" Germany) with a working frequency 70 MHz (internal standard – Me_4Si). According to ^{1}H-NMR, the level of isotopic purity of the growth medium was by 8–10 atom% lower than the isotope purity of the initial ^2H_2O.

Biosynthesis of [^{2}H]inosine

Biosynthetic [^{2}H]inosine was produced with an output 3,9 g/l in heavy water (HW) medium (89–90 atom% ^{2}H) using 2% (w/v) hydrolysate of deuterated biomass of the methanol assimilating strain of the facultative Gram-positive methylotrophic bacterium *Brevibacterium methylicum B-5662* as a source of ^{2}H-labeled growth substrates. The strain was obtained by multistage adaptation on a solid (2% (w/v) agarose) minimal salt M9 medium, containing 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 0,5 g/l NaCl, 1 g/l NH_4Cl and 2% (v/v) [^{2}H]methanol with a stepwise increasing gradient of ^2H_2O concentration (0; 24,5; 73,5, and 98% (v/v) ^2H_2O). Raw methylotrophic biomass (output, 200 g/l) 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 growing the inosine producer strain. For this purpose, an inoculum (5–6 % (w/v)) was added into HW medium with 99,8 atom% ^2H_2O containing 12% (w/v) glucose, 2% (w/v) hydrolysate of deuterated biomass of *B. methylicum B-5662*, 2% (w/v) NH_4NO_3, 1% (w/v) $MgSO_4$ $7H_2O$, 2% (w/v) $CaCO_3$, 0,01% (w/v) adenine, and 0,01% (w/v) uracil. As a control was used equivalent protonated medium containing 2% (w/v) yeast protein–vitamin concentrate (PVC).

Growth conditions

The bacterium was grown in 500 ml Erlenmeyer flasks (containing 100 ml of the growth medium) for 3–4 days at 32 °C under intensive aeration on a Biorad orbital shaker ("Biorad Labs", Hungary). The bacterial growth was controlled on the ability to form individual colonies on the surface of solid (2% (w/v) agarose) media with the same ^2H_2O-content, as well as on the optical density of the cell suspension measured on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) at λ = 540 nm in a quartz cuvette with an optical pathway length 10 mm.

Analytical determination of [^{2}H]inosine

Inosine was analytically determined in 10 μl of liquid culture (LC) samples on Silufol UV-254 chromatographic plates (150×150 mm) ("Kavalier", Czech Republic) using a standard set of ribonucleosides "Beckman-Spinco" (USA) in the solvent system: *n*-butanol–acetic acid–water (2:1:1, % (v/v)). Spots were eluted with 0,1 N HCl. The UV absorbance of eluates was recorded on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) using a standard calibration plot. The level of bioconversion of the carbon substrate was assessed using glucose oxidase (EC 1.1.3.4).

Isolation of [^{2}H]inosine from LC.

Samples of LC were separated on a T-26 centrifuge ("Carl Zeiss", Germany) at 2000 g for 10 min, concentrated at 10 mm Hg in a RVO-6 rotor evaporator ("Microtechna", Hungary) to half of their initial volume, and supplemented with acetone (3×5 ml). The mixture was kept for ~10 h at 4°C, and the precipitate was separated by centrifugation at 1200 g for 5 min. The supernatant was supplemented with 20 g of activated carbon and kept for 24 h at 4 °C. The water fraction was separated by filtration; the solid phase was supplemented with 20 ml 50% (v/v) EtOH solution in 25% (v/v) ammonia (1:1, (v/v)) and heated at 60°C with a reflux water condenser. After 2–3 h, the mixture was filtered and evaporated at 10 mm Hg. The product was extracted with 0,3 M ammonium–formate buffer (pH = 8,9), washed with acetone (2×10 ml), and dried over anhydrous $CaCl_2$. Inosine was crystallized from 80% (v/v) ethanol ($[\alpha]_D^{20}$ = +1,61°; output, 3,1 g/l (80 %)). Inosine was finally purified by ion exchange chromatography using a calibrated column (150 × 10 mm) with AG50WX 4 cation exchange resin ("Pharmacia", USA). The column was equilibrated with 0,3 M ammonium–formate buffer (pH = 8,9) containing 0,045 M NH_4Cl and eluted with the same buffer under isocratic conditions (chromatographic purity, 92%). The eluate was dried in vacuum and stored in sealed ampoules at -14°C in frost camera. ^{2}H-inosine: yield – 3,1 g/l (80%); T_m = 68–70 °C; $[\alpha]_D^{20}$ = 1,61 (ethanol); R_f = 0,5; pK_a = 1,2 (phosphate buffer with pH = 6,87). UV-spectrum (0.1 N HCl): λ_{max} = 249 nm; ε_{249} = 7100 $M^{-1} \cdot cm^{-1}$. FAB mass spectrum (glycerol matrix, Cs^+; accelerating voltage, 5 kV; ion current – 0,6–0,8 mA): $[M + H]^+$ m/z (I, %) 273, 20% (4 atoms ^{2}H); 274, 38 % (5 atoms ^{2}H); 275, 28% (6 atoms ^{2}H); 276, 14% (7 atoms ^{2}H); $[A + H]^+$ 136, 46%; $[B + H]^+$ 138, 55%; $[B – HCN]^+$ 111, 49%; $[B – HCN]^+$ 84, 43%.

Protein hydrolysis

Dry biomass (10 g) was treated with a chloroform–methanol–acetone mixture (2:1:1, % (v/v)), evaporated in vacuum, and supplemented with 5 ml 6 N ^{2}HCl (in ^2H_2O). The ampules were kept at 110 °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. For preparation of ^{2}H-labeled growth substrates 200 mg of raw deuteron-biomass was suspended in 200 ml 0,5 ^{2}HCl (in ^2H_2O) and autoclaved at 60 °C for ~1,5 h. The reaction mixture was neutralized with 0,5 N NaOH (in ^2H_2O) till pH = 6,5–6,7, and evaporated at 10 mm Hg. The dry residue was used for preparation of growth media.

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 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 Biotronic LC-5001 (230×3,2 mm) column ("Eppendorf–Nethleler–Hinz", Germany) with UR-30 sulfonated styrene resin ("Beckman–Spinco", USA) as a stationary phase; the mobile phase – 0,2 N sodium–citrate buffer (pH = 2,5); the granule diameter – 25 μm; working pressure – 50–60 atm; the eluent input rate – 18,5 ml/h; the ninhydrin input rate – 9,25 ml/h; detection at λ = 570 and λ = 440 nm (for proline).

Analysis of carbohydrates

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

UV spectroscopy

The UV spectra were registered with Beckman DU-6 programmed spectrophotometer ("Beckman Coulter", USA) at λ = 220–280 nm.

FAB mass spectrometry

FAB mass spectra were recorded on VG-70 SEQ chromatograph ("Fisons VG Analytical", USA) equipped with a cesium source on a glycerol matrix with accelerating voltage 5 kV and ion current 0,6–0,8 mA.

EI mass spectrometry

EI mass spectra were recorded with 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 °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.

Results and discussion

Preparation of Deutero-Biomass of B. methylicum

For this study was used a mutant strain of the Gram-positive chemoheterotrophic bacterium *B. subtilis B-3157*, polyauxotrophic for histidine, tyrosine, adenine, and uracil (preliminary adapted to deuterium by selection of individual colonies on growth media with increased ^2H_2O content). Due to impaired metabolic pathways involved in the regulation of the biosynthesis of purine ribonucleosides, this strain under standard growth conditions (PVC medium, late exponential growth, 32 °C) synthesizes 17–20 g of inosine per 1 liter of LC [21].

The maximal yield of inosine was attained on a protonated medium with 12% (w/v) glucose as a source of carbon and energy and 2% (w/v) yeast PVC as a source of growth factors and amine nitrogen. In our experiments it was necessary to replace the protonated growth substrates with their deuterated analogs, as well as to use ^2H_2O of high isotopic purity. For this purpose, we used autoclaved biomass of the Gram-positive facultative methylotrophic bacterium *B. methylicum B-5662*, capable to assimilate methanol *via* RuMP pathway of carbon assimilation. Owing to a 50–60% rate of methanol bioconversion (conversion efficiency – 15,5–17,3 gram dry biomass per 1 gram of assimilated substrate) and stable growth on deuterated minimal medium M9 with 98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol, this strain is the most convenient source for producing the deuterated biomass; moreover, the cost of bioconversion is mainly determined by the cost of ^2H_2O and [^{2}H]methanol [22].

Adaptation of *B. methylicum B-5662* was necessary to improve the growth characteristics of this strain and attain high output of microbial biomass on the maximally deuterated M9 medium. For this purpose, we used a stepwise increasing gradient of ^2H_2O-concentration in M9 growth media (from 24,5; 49,0; 73,5 up to 98% (v/v) ^2H_2O) in the presence of 2% (v/v) methanol and its ^{2}H-labeled analog ([^{2}H]methanol), because we assumed that gradual cell adaptation to ^2H_2O would have a favorable effect on the growth parameters of the strain.

To study the effect of the degree of carbon source deuteration on the growth parameters of the strain, in experiments 1, 3, 5, 7, and 9 was used protonated methanol, and [^{2}H]methanol in experiments 2, 4, 6, 8, and 10 (Table 1). The results demonstrated that the replacement of protonated methanol with its deuterated analog within the same concentration of ^2H_2O in the growth media slightly decreased the growth characteristics (Table 1, experiments 2, 4, 6, 8, and 10). Therefore, in further experiments were used M9 media with ^2H_2O and [^{2}H]methanol. When the initial strain of *B. methylicum* was cultivated on protonated M9 medium with water and methanol, the duration of lag-phase and cell generation time were 20 and 2,2 h, respectively, with an output of biomass 200 gram per 1 liter of LC (Table 1, experiment 1). In the intermediate experiments (2–10), these parameters varied proportionally to the ^2H_2O concentration (Table 1). The observed effect consisted in the increase in the lag-phase period and cell generation time with a simultaneous decrease in microbial biomass outputs on media with increasing ^2H_2O-content. The most remarkable values of this parameters were detected in experiment 10, in which was used the maximally deuterated medium with 98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol; the lag-phase

and cell generation time in these conditions were increased in 3- and 2,2-fold times, respectively, as compared to the control conditions (water and methanol; Table 1, experiment 1), and the biomass output decreased in 3,1-fold. The adaptation to deuterium (experiment 10', Table 1) permitted to improve essentially the growth characteristics of *B. methylicum B-5662* on maximally deuterated growth medium. The output of biomass produced by the adapted bacterium decreased by 13% as compared to the control with an increase in the generation time to 2,8 h and the lag phase to 40 h (experiment 10', Table 1).

Table 1: Isotopic components of growth media M9 and characteristics of bacterial growth of *B. methylicum B-5662**

Experiment number	Media components, % (v/v)				Lag-period (h)	Yield of biomass, gram from 1 l of LC	Generation time (h)
	H_2O	^2H_2O	Methanol	[^{2}H]methanol			
1	98,0	0	2	0	20±1,40	200,2±3,20	2,2±0,20
2	98,0	0	0	2	30±1,44	184,6±2,78	2,4±0,23
3	73,5	24,5	2	0	32±0,91	181,2±1,89	2,4±0,25
4	73,5	24,5	0	2	34±0,89	171,8±1,81	2,6±0,23
5	49,0	49,0	2	0	40±0,90	140,2±1,96	3,0±0,32
6	49,0	49,0	0	2	44±1,38	121,3±1,83	3,2±0,36
7	24,5	73,5	2	0	45±1,.41	112,8±1,19	3,5±0,27
8	24,5	73,5	0	2	49±0,91	94,4±1,74	3,8±0,25
9	0	98,0	2	0	58±1,94	65,8±1,13	4,4±0,70
10	0	98,0	0	2	60±2,01	60,2±1,44	4,9±0,72
10'	0	98,0	0	2	40±0,88	174,0±1,83	2,8±0,30

Notes: * The data in Expts. 1–10 are submitted for *B. methylicum* at growing on growth media with 2% (v/v) methanol/[^{2}H]methanol and specified amounts (%, v/v) ^2H_2O. The data in Expt. 10' are submitted for adapted for maximum content of deuterium in growth medium bacterium *B. methylicum* at the growing on growth media with 2% (v/v) of [^{2}H]methanol and 98% (v/v) of ^2H_2O. As the control used experiment where used ordinary protonated water and methanol.

The adaptation was monitored by recording the growth dynamics of the initial bacterium (Figure 1, curve *1*, control, protonated M9 medium) and adapted to deuterium *B. methylicum B-5662* (Figure 1, curve *3*) on the maximally deuterated M9 medium with 98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol. Unlike the adapted bacterium (Figure 1, curve *3*), the growth dynamics of the initial bacterium (Figure 1, curve *1*) on the maximally deuterated medium were inhibited by deuterium. Being transferred to the protonated medium, the adapted bacterium returned to normal growth after a certain lag-phase period, that was characteristic for other adapted bacterial strains. The effect of growth reversion in protonated/deuterated media demonstrates that adaptation to ^2H_2O is a phenotypic phenomenon, although it cannot be excluded that a certain genotype determined the manifestation of the same phenotypic attribute in media with high deuterium content. In general, the improved growth characteristics of the adapted bacterium significantly simplify the scheme for the production of deuterated biomass, the optimal conditions for which are satisfied the following: maximally deuterated M9 medium with 98% (v/v) ^2H_2O and 2% (v/v) [^{2}H]methanol, incubation period 3–4 days, and temperature 35 ^{o}C.

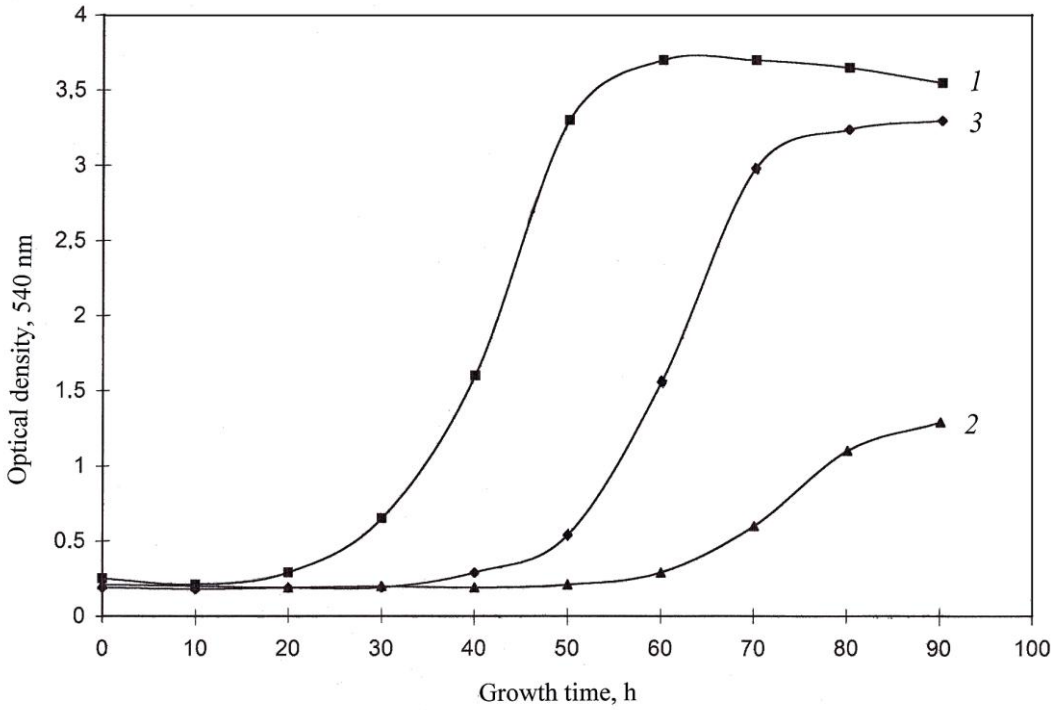


Figure 1. Growth dynamics of *B. methylicum B-5662* (1, 2, 3) on media M9 with various isotopic content: *1* – non-adapted bacterium on protonated medium M9 (Table 1, experiment 1); *2* – non-adapted bacterium on maximally deuterated medium M9 (Table 1, experiment 10); *3* – adapted to ^2H_2O bacterium on maximally deuterated medium M9 (Table 1, experiment 10')

Biosynthesis of [²H]Inosine.

The strategy for the biosynthesis of [²H]inosine using biomass of *B. methylicum B-5662* as growth substrates was developed taking into account the ability of methylotrophic bacteria to synthesize large amounts of protein (output, 50% (w/w) of dry weight), 15–17% (w/w) of polysaccharides, 10–12% (w/w) of lipids (mainly, phospholipids), and 18% (w/w) of ash [23]. 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).

Since the inosine-producing strain of *B. subtilis B-3157* is a polyauxotroph requiring tyrosine and histidine for its growth, we studied the qualitative and quantitative content of amino acids in the hydrolyzed methylotrophic biomass produced in the maximally deuterated medium M9 with 98% (v/v) ^2H_2O and 2% (v/v) [²H]methanol, and the levels of their deuterium enrichment (Table 2). The methylotrophic hydrolysate 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 3,72% (w/w), thereby satisfying the auxotrophic requirements of the inosine producer strain for these amino acids. 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 (Table 2).

Table 2**:** Amino acid composition of hydrolyzed biomass of the facultative methylotrophic bacterium *B. methylicum B-5662* 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***
	Protonated sample (control)	Sample from deuterated M9 medium		
Glycine	8,03	9,69	2	90,0±1,86
Alanine	12,95	13,98	4	97,5±1,96
Valine	3,54	3,74	4	50,0±1,60
Leucine	8,62	7,33	5	49,0±1,52
Isoleucine	4,14	3,64	5	49,0±1,50
Phenylalanine	3,88	3,94	8	95,0±1,85
Tyrosine	1,56	1,83	7	92,8±1,80
Serine	4,18	4,90	3	86,6±1,56
Threonine	4,81	5,51	–	–
Methionine	4,94	2,25	–	–
Asparagine	7,88	9,59	2	66,6±1,62
Glutamic acid	11,68	10,38	4	70,0±1,64
Lysine	4,34	3,98	5	58,9±1,60
Arginine	4,63	5,28	–	–
Histidine	3,43	3,73	–	–

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.

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 49 atom% ^{2}H for leucine/isoleucine to 97,5 atom% ^{2}H for alanine (Table 2). This allowed using the hydrolysate of deuterated biomass of *B. methylicum* as a source of growth substrates for growing the inosine-producing strain *B. subtilis*.

The growth and biosynthetic characteristics of inosine-producing strain *B. subtilis B-3157* 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) of hydrolysate of deuterated biomass of *B. methylicum* (Figure 2). Experiments demonstrated a certain correlation between the changes of growth dynamics of *B. subtilis B-3157* (Fig. 2, curves *1*, *1*'), output of inosine (Figure 2, curves *2*, *2*'), and glucose assimilation (Figure 2, 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 2, curve *2*). The output of inosine in the HW medium decreased in 4,4-fold, reaching 3,9 g/l (Figure 2, curve *2*'), and the level of glucose assimilation – 4-fold, as testified by the remaining 40 g/l non-assimilated glucose in LC (Figure 2, 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.

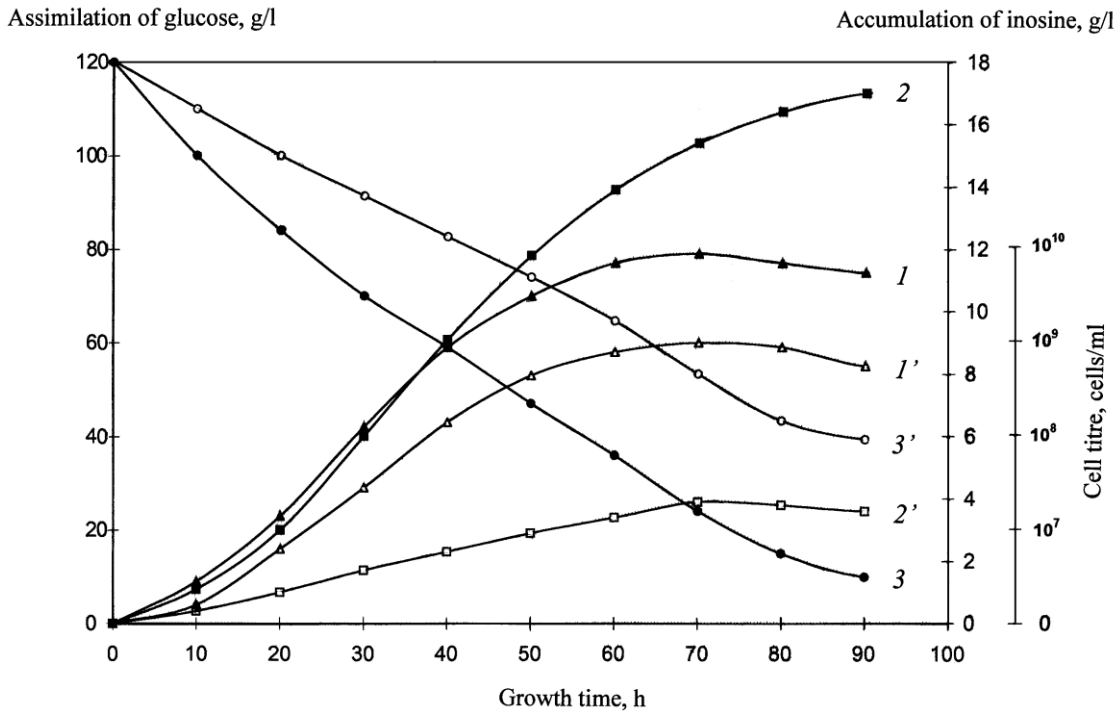


Figure 2. Growth dynamics of *B. subtilis B-3157* (*1*, *1*') (cells/ml), inosine accumulation in LC (*2*, *2*') (g/l), and glucose assimilation (*3*, *3*') (g/l) under different experimental conditions: (*1–3*) – protonated yeast PVC medium; (*1*'–*3*') – HW medium with 2% (w/v) hydrolysate of deuterated biomass of *B. methylicum B-5662*.

This result demanded the examination of the content of glucose and other intracellular carbohydrates in the biomass of the inosine-producer strain of *B. subtilis B-3157*, which was performed by reverse phase HPLC on an Ultrasorb CN column (10 μm, 10×250 mm) with a mixture of acetonitrile–water (75:25, % (v/v)) as a mobile phase (Table 3). The fraction of intracellular carbohydrates in Table 3 (numbered according to the sequence of their elution from the column) comprises monosaccharides (glucose, fructose, rhamnose, and arabinose), disaccharides (maltose and sucrose), and four unidentified carbohydrates with retention times of 3,08 (15,63% (w/w)), 4,26 (7,46% (w/w)), 7,23 (11,72% (w/w)), and 9,14 (7,95% (w/w) min (not shown). As was expected, the output of glucose in the deuterated hydrolysate was 21,4% (w/w) of dry weight, that is, higher than the outputs of fructose (6,82% (w/w)), rhamnose (3,47% (w/w)), arabinose (3,69% (w/w)), and maltose (11,62% (w/w)) (Table 3). Their outputs in microbial biomass did not differ considerably related to the control in H_2O except for sucrose, which is undetectable in the deuterated sample. The levels of deuterium enrichment in carbohydrates varied from 90,7 atom% ^2H for arabinose to 80,6 atom% ^2H for glucose.

Table 3: Qualitative and quantitative compositions of intracellular carbohydrates isolated from *B. subtilis B-3157* after growing on HW-medium and levels of the deuterium enrichment*

Carbohydrate	Content in biomass, % (w/w) of 1 g of dry biomass		Level of deuterium enrichment of molecules, %**
	Protonated sample (control)	Sample from the HW medium	
Glucose	20,01	21,40	80,6±1,86
Fructose	6,12	6,82	85,5±1,92
Rhamnose	2,91	3,47	90,3±2,12
Arabinose	3,26	3,69	90,7±3,10
Maltose	15,30	11,62	–
Sucrose	8,62	ND**	–

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

Isolation of [²H]Inosine from LC.

The use of a combination of physical-chemical methods for isolating [²H]inosine from LC was determined by the need for preparing inosine of a high chromatographic purity (no less than 95%). Since LC along with inosine contains inorganic salts, proteins, and polysaccharides, as well as accompanying secondary nucleic metabolites (adenosine and guanosine) and non-reacted substrates (glucose and amino acids), LC was fractionated in a stepwise manner for isolating of [²H]inosine. The high sensitivity of inosine to acids and alkali and its instability during isolation required applying diluted acid and alkaline solutions with low concentration, as well as carrying out the isolation procedure at low temperature, thus avoiding long heating of the reaction mixture. The fractionation of LC 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 LC by precipitation with acetone at 4 °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 °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 3 shows UV-absorption spectra of inosine isolated from the LC of *B. subtilis B-3157* 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 3, curve *3*), demonstrates the homogeneity of isolated product and the efficiency of the isolation method.

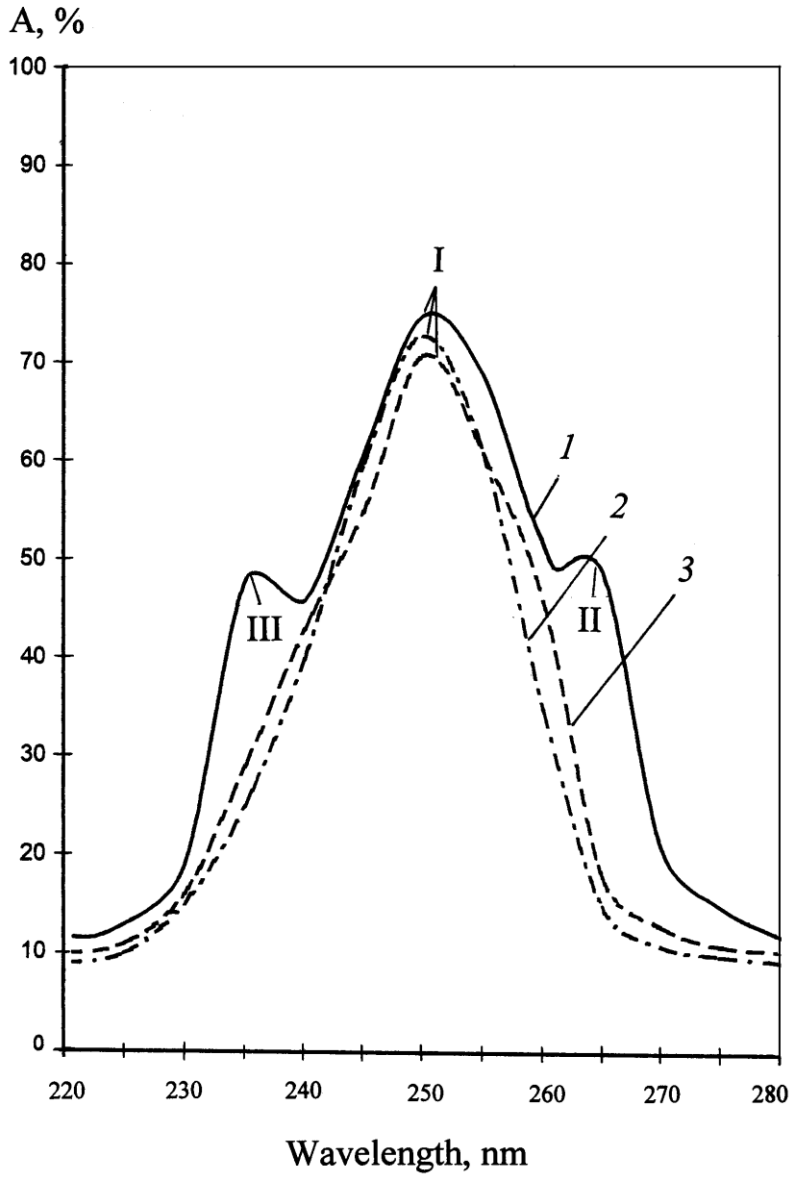


Figure 3. UV-absorption spectra of inosine (0.1 N HCl): (*1*) – initial LC after the growth of *B. subtilis B-3157* on HW medium; (*2*) – natural inosine; (*3*) – inosine extracted from the LC of *B. subtilis B-3157*. Natural inosine (*2*) was used as a control: (I) – inosine, (II, III) – secondary metabolites.

The Studying of the Level of Deuterium Enrichment of [^{2}H]Inosine.

The level of deuterium enrichment of the [^{2}H]inosine molecule 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 [24]. 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 4*b* regarding the control (natural protonated inosine, Figure 4*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 4*a*). That result corresponds to five deuterium atoms incorporated into the inosine molecule, obtained after growing of *B. subtilis B-3157* on HW-medium (Figure 4b). 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 biosynthetic inosine isolated from HW-medium

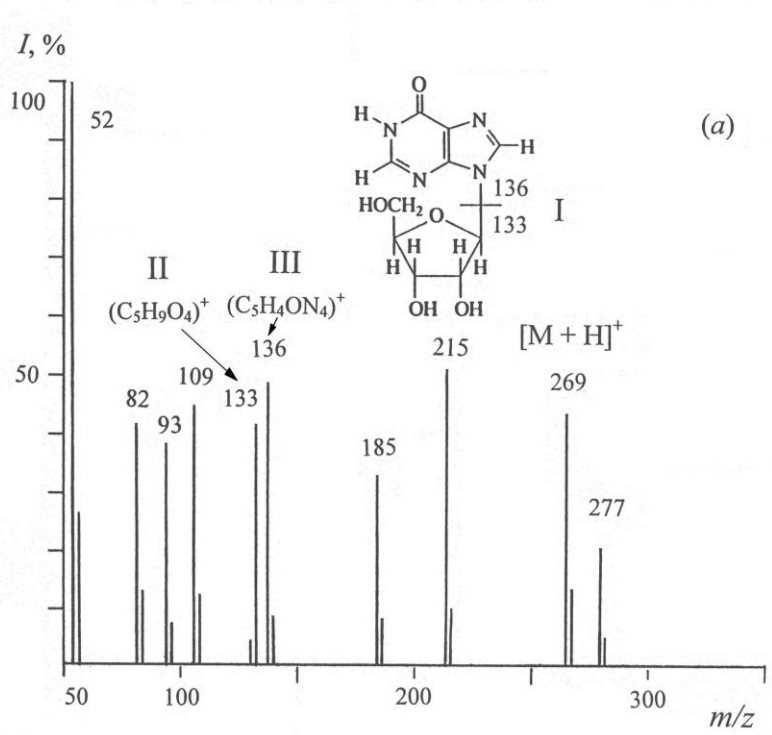
Value of peak $[M+H]^+$	Contribution to the level of deuterium enrichment, mol.%	The number of deuterium atoms	Level of deuterium enrichment of molecules, % of the total number of hydrogen atoms*
273	20	4	20,0±0,60
274	38	5	62,5±1,80
275	28	6	72,5±1,96
276	14	7	87,5±2,98

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:

$$TLDE = \frac{[M]^+_{r1} \cdot C_2 + [M]^+_{r2} \cdot C_2 + ... + [M]^+_{rn} \cdot C_n}{\sum C_n}, \qquad (1)$$

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



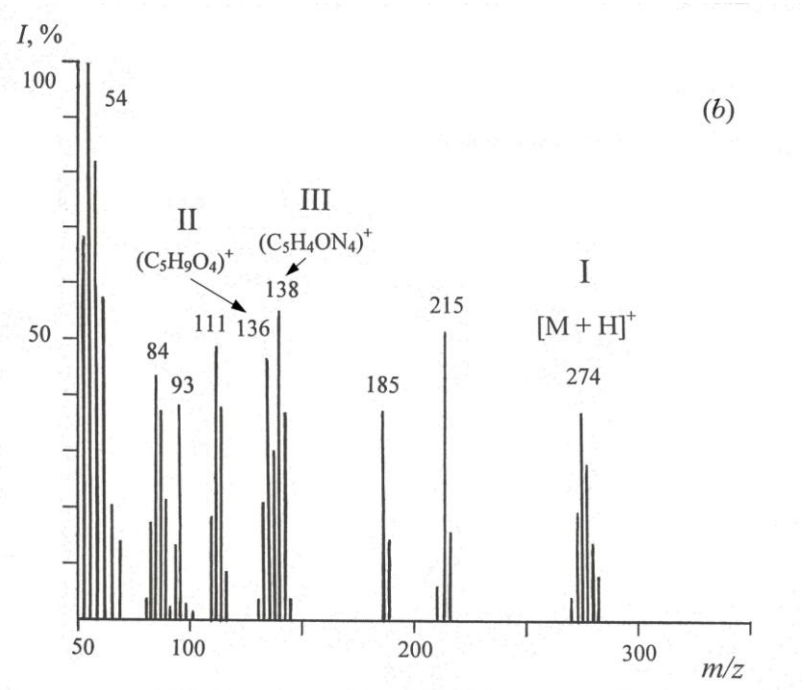


Figure 4. FAB mass spectra of inosine (glycerol as a matrix) under different experimental conditions: (*a*) – natural inosine; (*b*) – [^{2}H]inosine isolated from HW medium (scanning interval at *m/z* 50–350; major peaks with a relative intensity of 100% at *m/z* 52 and *m/z* 54; ionization conditions: 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 5, 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 5). 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 5). 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.

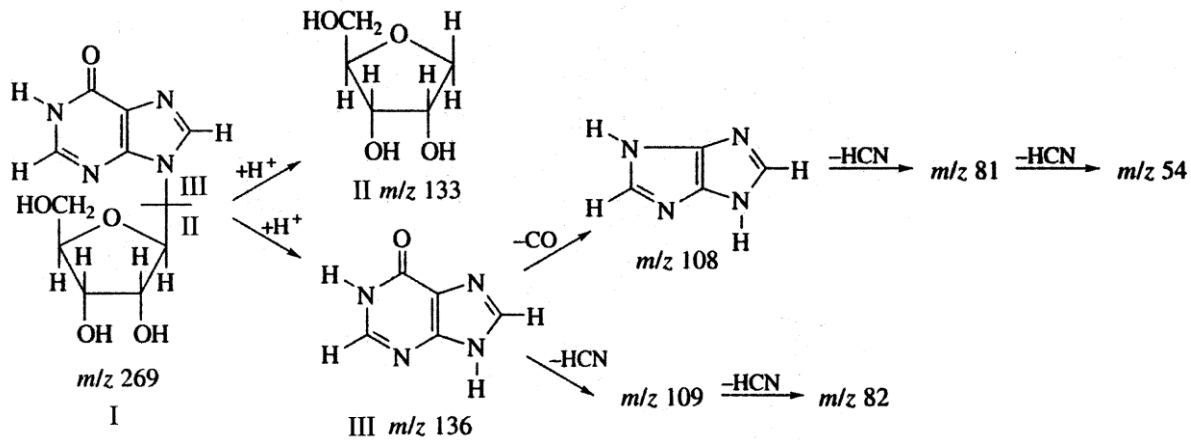


Figure 5. The fragmentation pathways of the inosine molecule leading to formation of smaller fragments by the FAB-method

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. The carbon source was glucose as a main substrate and a mixture of deuterated amino acids from deuterated hydrolizate of methylotrophic bacterium *B. methylicum B-5662* as a source of deuterated substrated 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 hexose-5-monophosphate (HMP) shunt (Figure 6), associated with the assimilation of glucose and other carbohydrates. HMP shunt is a complex of 12 reversible enzymatic reactions resulting in the oxidation of glucose to CO_2 to form reduced NADPH, and H^+, and the synthesis of phosphorylated sugars containing from 3 to 7 carbon atoms. 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 the preservation in this bacterium the minor pathways of *de novo* glucose biosynthesis in ^2H_2O-medium. It became possible that the cell uses its own resources for intracellular biosynthesis of glucose from intracellular precursors.

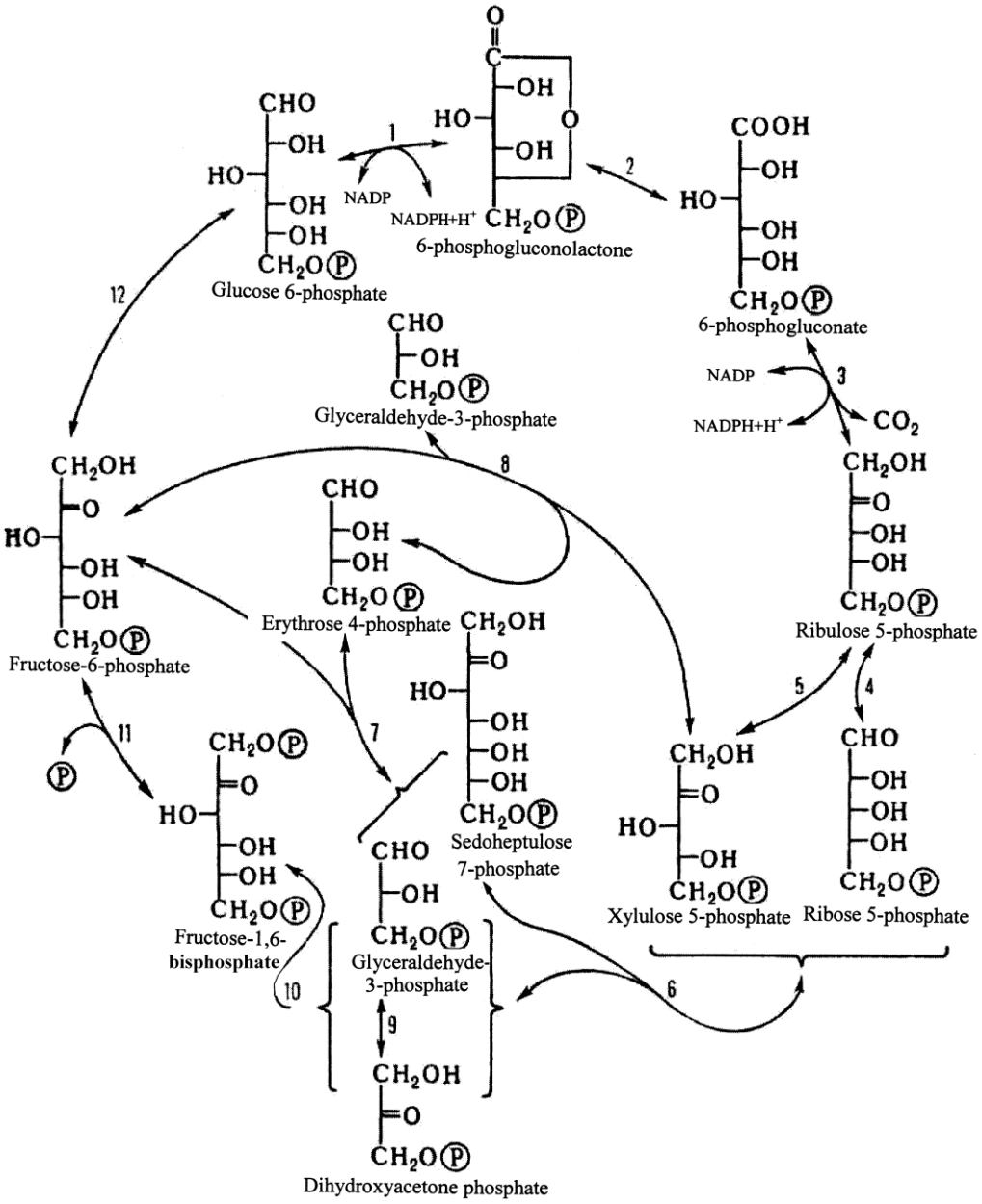


Figure 6. Scheme of hexose monophosphate shunt of glucose oxidation (adapted from): 1 – glucose-6-phosphate dehydrogenase; 2 – 6-phosphogluconolactonase; 3 – 6-phosphogluconate dehydrogenase; 4 – ribulose 5-phosphate isomerase; 5 – ribulose-5-phosphate 3-epimerase; 6 – transketolase; 7 – transaldolase; 8 – transketolase; 9 – triose phosphate isomerase; 10 – aldolase; 11 – fructose 1,6-bisphosphatase; 12 – hexose-6-phosphate isomerase. Total reaction: 6 glucose-6-phosphate + 12 NADP → 5 glucose-6-phosphate + 6 CO_2 + 12 NADPH +12 H^+ + $H_2PO_4^-$ (adapted from L. Stryer [25])

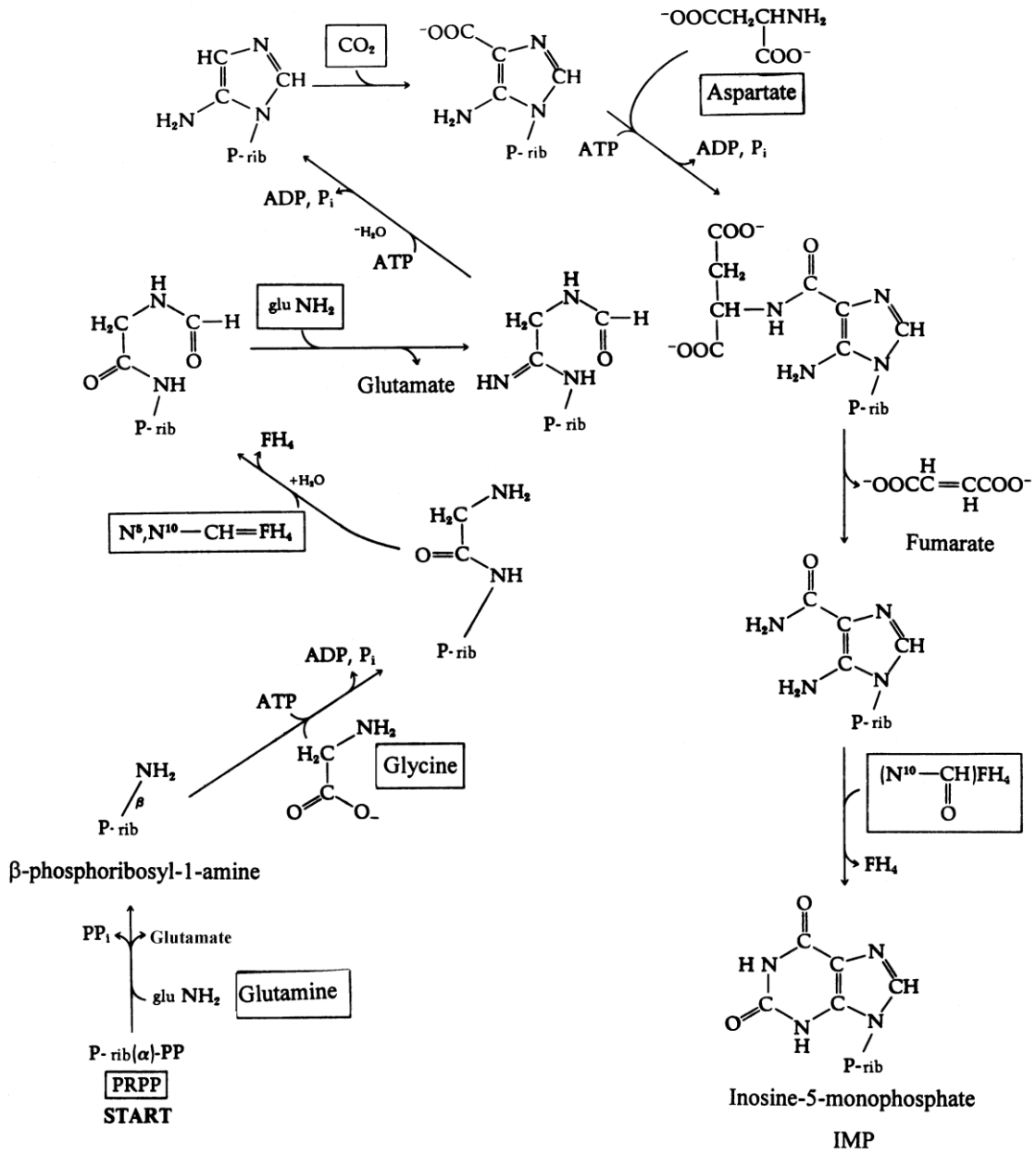


Figure 7. Scheme of biosynthesis of IMP by microbial cell (adapted from Bohinski, 1983 [26])

It should be noted that numerous isotopic $^{1}H–^{2}H$ 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 $^{2}H_2O$ *via* keto–enol tautomerism. Three non-exchangeable deuterium atoms in the ribose residue of inosine are synthesized *de novo* and could have been originated from HMP shunt reactions, 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 (Figure 7), that originated from deuterated hydrolysate of methylotrophic bacterium *B. methylicum* obtained on 98% of $^{2}H_2O$ medium. In particular, the glycoside proton at β-N_9-glycosidic bond could be replaced with deuterium *via* the reaction of CO_2 elimination at the stage of ribulose-5-monophosphate formation from 3-keto-6-phosphogluconic acid with subsequent proton (deuteron) attachment at the C1-position of ribulose-5-monophosphate (Figure 7). Two other protons at C2(C3) and C4 positions in ribose residue could be replaced with deuterium *via* further enzimatic isomerization of ribulose-5-

monophosphate into ribose-5-monophosphate. In general, our studies confirmed this scheme [27]. However, it should be noted that the level of deuterium enrichment of 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*.

Conclusion

We have demonstrated the feasibility of using the FAB method for studying of biosynthetic pathways of biosynthisis of ^2H-labeled inosine by the bacterium *Bacillus subtilis B-3157* and evaluation of deuterium incorporation into the inosine molecule. For this aim [^2H]inosine was isolated from HW-medium by adsorption/desorption on activated carbon, extraction by 0,3 M ammonium–formate buffer (pH = 8,9), crystallization in 80% (v/v) EtOH, and IEC on AG50WX 4 cation exchange resin equilibrated with 0,3 M ammonium–formate buffer and 0,045 M NH_4Cl with output 3,9 g/l. The total level of deuterium enrichment of the inosine molecule was 5 deuterium atoms (65,5 atom% ^2H). From total 5 deuterium atoms in the inosine molecule, 3 deuterium atoms were localized in the ribose residue, while 2 deuterium atoms – in the hypoxanthine residue of the molecule. Deuterium was incorporated into the ribose residue of the inosine molecule owing to the preservation in this bacterium the minor pathways of *de novo* glucose biosynthesis in ^2H_2O-medium. Three non-exchangeable deuterium atoms in the ribose residue of inosine were synthesized *de novo* and originated from HMP shunt reactions, while two other deuterium atoms at C2,C8-positions in the hypoxanthine residue could be synthesized *de novo* from [^2H]amino acids, that originated from deuterated hydrolysate of *B. methylicum B-5662* obtained on 98% of ^2H_2O medium. To attain higher deuterium enrichment level of the final product, it is necessary to thoroughly control the isotope composition of the growth medium and exclude any possible sources of additional protons, in particular, to use [^2H]glucose, which may be isolated from deuterated biomass of the methylotrophic bacterium *B. methylicum B-5662*.

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Virtual Screening SNP-Polymorphisms of Genes Determining the High Level of General Non-Specific Reactivity of Organism

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Abstract

As a result of a bioinformatic search using resources of NCBI PubMed Central, PDB, KEGG, and SNP authors have developed a database of genes associated with phenotypic manifestations of general non-specific reactivity level (GNRL). Out of 164 genes primarily relevant by search criteria for a detailed analysis there are selected 23 genes, divided into four groups: genes associated with the synthesis and reception of neurotransmitters (1); genes associated with membrane transport of electrolytes (2); genes associated with the synthesis of interleukins (3); and genes associated with certain metabolic response (4). After studying the SNP-polymorphisms annotations in the database NCBI-SNP, 10 genes and 20 SNP- polymorphisms were identified as the most likely candidates for the potential formation of phenotypic manifestations for GNRL. Further analysis of the degree of influence the conformational variability of amino acid chains in forming the secondary structure of proteins on their likely functional properties allows to select as promising the next 6 SNP: rs1851048 and rs 6777055 in the *cacna2d3* gene, encoding the voltage gated Ca^{2+} channels; rs2562456 in *znf-ld* gene of zinc-containing transcriptional regulator of DNA methylation; rs6923492 and rs362962 in *grm1* gene of metabotropic glutamate receptor; and rs6314 in *htr2a* gene, coding for serotonin receptor type 2A.

Keywords: pain, general non-specific reactivity of an organism, genetics, SNP-polymorphisms, bioinformatics.

Introduction

In earlier studies it was justified a property of integrative manner for general nonspecific reactivity level (GNRL) of the organism, which manifests itself in a functional unity of all organism systems through the central coordination of the extent of their sensitivity, reactivity and activity [1-3]. Currently the GNRL actively is used as a criterion for estimation of predicting and modulation of the functional states of the human organism and animal species [4-6].

For the further research in this direction it must be better detailed the study of the genetic prerequisites for the GNRL formation. As the universal indicator of the GNRL in the animals experiment and in the human study is a pain threshold, the problem of genetic determinism of the GNRL, at least initially, can be reduced to the search for genes associated with phenotypic variability of pain sensitivity.

By its biological origin, pain is one of the most important signals that inform organism about the damage of tissues and trigger a whole range of defensive reactions designed to minimize the and eliminate the damage [7]. Genetic factors contribute significantly to individual differences in the tolerance thresholds and psychophysical perception of painful sensation during the formation of responses to pain stimuli of different nature. The spread of individual differences in pain sensitivity is a challenging task for medical diagnostics and may have a separate value in the mechanisms of the chronic pain syndromes development [8-10].

The genetic approach to the pain study by use of model organisms identified the molecular nature of the nociceptive stimulus transformation, regulatory mechanisms associated with changes in neuronal activity, and the important role of immune system cells in the nociceptive pathways stimulation.

In humans, the genetic contribution to the formation of pain sensitivity is the most studied through the use of twin method, by comparing the ratio of the pain threshold between monozygotic and dizygotic twins. It was received the confirmation of the genetic contribution to sensitivity to the most painful stimuli. The observations showed that consanguineous marriages cause changes in pain threshold [11]. So, it was revealed the predominant genetic determinancy of painful sensation in regard to the cold - 60% compared to 26% of the determinism of the pain threshold to the heat perception [12]. In addition to experimentally induced pain, several studies have been devoted to the study of the genetic variation contribution in severity and susceptibility to pain at chronic diseases [13-15]. It was found that in formation of chronic pain syndrome a determining factor is genetically determined reactivity of the organism (especially structures of central nervous system) [7]. Twin studies demonstrated heritability from 52% to 68% of painful sensations in the back and from 35% to 58% of pain in the neck, which proves the genetic contribution to formation of the nociceptive sensation [9].

The complexity of human studies consists in the difficulty of creating similar environmental living conditions, phenotypic and genotypic homogeneity of the research objects. In this regard, animal experiments are important component in the study of genetic mechanisms in pain and analgesia. Experimental animal studies are strong evidence of pain and analgesia heritability [10, 16], the correlation between the pain threshold and psycho-emotional status [17].

Currently, it is known, at least 23 genes associated with experimentally induced pain, clinical pain or anesthesia [10]. Studies on the inbred line of mice, suggest that there are at least five genetically different basic types of nociception and hypersensitivity: basic thermal nociception (1), spontaneous reactions to noxious chemical stimuli (2) thermal sensitivity (3) mechanical hypersensitivity (4), and the afferent input of independent hypersensitivity (5). At the same time, variations genes that regulate the processing of nociceptive stimuli at various levels of the nervous system, may affect the perception of several types of stimuli. Thus, in addition to the system of cellular perception of nociceptive stimuli it is possible that a single gene can affect the coding and processing of multiple types of stimuli [18]. Thus, it is likely the existence of multiple genetic variants of the perception of painful sensation [19, 20].

Objective

To create a database of genes involved in the formation of the level of general nonspecific reactivity of the human organism, taking into account the phenomenon of point polymorphisms and select based on virtual screening genes, the most promising for the establishment of an appropriate diagnostic kit.

Material and Methods

In carrying out the bio information retrieval, to create a structured and easy-to-use database it was searched and analyzed the information in open sources: e-source NCBI, PubMedCentral, PDB, KEGG and SNP. The search is performed by following keywords: pain, pain threshold, nociception, and the specific genes in combination with a single nucleotide polymorphism, gene polymorphism. The analysis has been selected more than 400 sources for 10-year period.

For the database design, we chose shell Microsoft Access, the layout of the database was obtained using Microsoft Visio Program ("Microsoft Corp"., USA). To build the database were used the following criteria: adequacy, completeness, stability. Given these criteria, were formed the following fields to the database: the full name of the gene, standard abbreviations and commonly used synonyms, a direct function of the encoded protein, the presence of polymorphisms, functional annotation of SNPs (impact on the direct function of the encoded protein), a functional effect on the reactivity (in the including probability) sources.

Results and Discussion

As a result of the analysis and construction was obtained the following data base (Fig. 1).

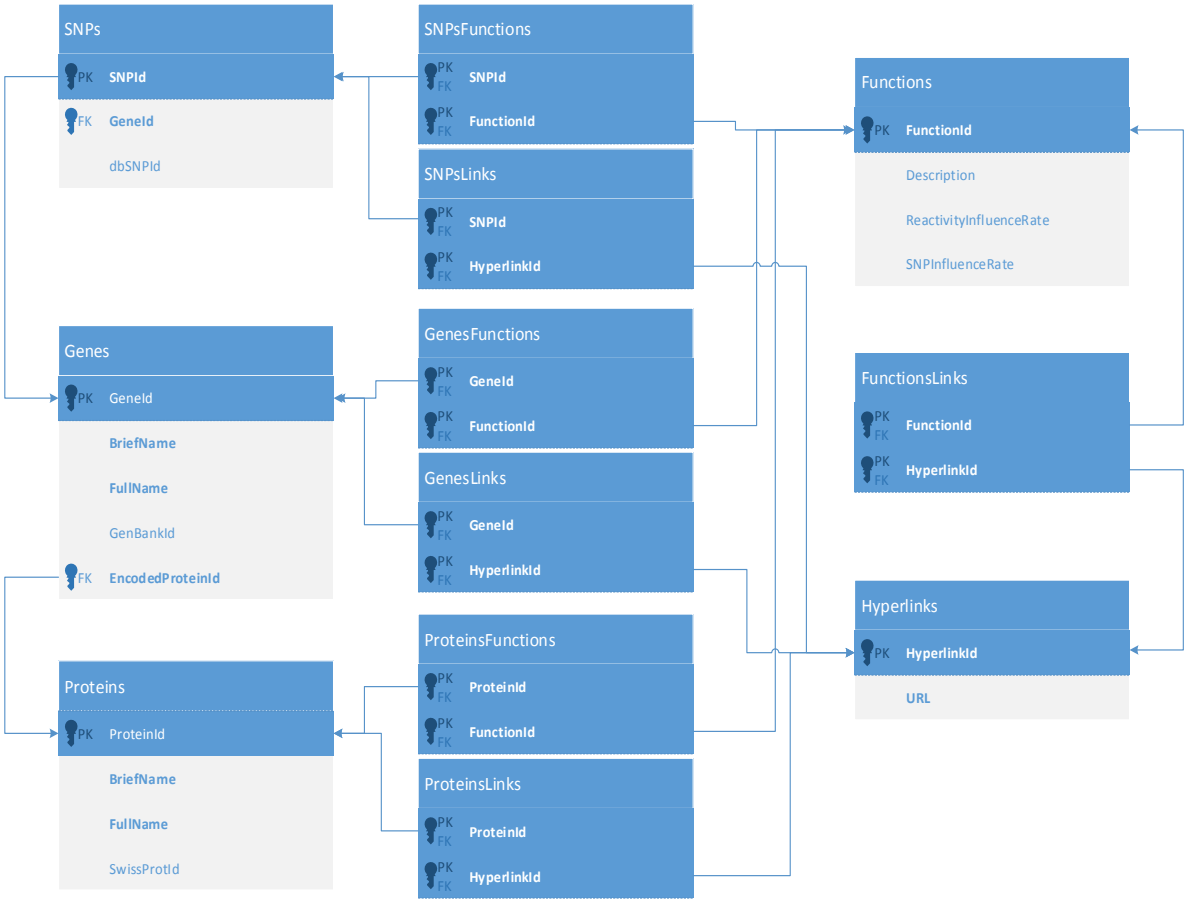

Figure 1. A working block diagram of a database of genes associated with phenotypic characteristics of pain perception in the shell of MS Vision

As a result, in the database are recorded 164 genes from the human genome that met the combination of characteristics: the proven facts of pain perception variations until the increased frequency of various chronic pain syndromes (1) and the availability of the annotated SNPs associated with these phenotypic variations (2). After exclusion of one-time mention, which did not find confirmation in future studies, and cases of describing rare inherited syndromes or cases of detection of variation only in situations of severe visceral pain (cancer surgery, traumatic operational effects, etc.) for the subsequent analysis were chosen 23 major genes (Table 1).

Table 1: A list of major genes associated with phenotypic characteristics of pain perception and reactive response of the human organism to the pain perception

Gene	Encoded Protein	Variation of Phenotype	Ref
The genes associated with the synthesis and reception of neurotransmitters			
adrb2	Adrenergic receptor β2	Visceral pain, vascular reactions	[21-23]
comt	Catechol-O-methyltransferase	Somatic and visceral pain	[15,24-26]
gch1	GTP cyclohydrolase 1	The exchange of biogenic amines, somatic and visceral pain	[14, 27, 28]
grm1	Glutamate receptor 1	Pain sensitivity and tolerance of pain	[29]
hcrtr2	Orexin B	Headache	[30, 31]
htr2a	Serotonin receptor 2A	Chronic pain syndromes	[32, 33]
mc1r	Melanocortin-1 receptor	Experimental pain, dependent conditions	[26]
oprd1	Opioid receptor, delta 1	Experimental pain	[34]
oprm1	Opioid receptor, mu 1	Experimental pain, dependent conditions	[35-37]
p2rx7	Ca^{2+}-permeable cationic channels	Chronic pain syndromes	[38]
slc6a4	Serotonin transporter	Chronic pain syndromes, experimental pain	[39, 40]
trpa1	Transient receptor potential A1	Experimental pain	[34]
trpv1	Transient receptor potential V1	Experimental pain	[34]
Genes associated with membrane transport of electrolytes			
cacna1a	P/Q type neuronal calcium channel	Headache	[30]
cacna2d3	Voltage gated Ca^{2+} channels	Chronic pain syndromes, experimental pain	[41, 42]
kcns1	Voltage gated K^+ channels	Chronic pain syndromes, experimental pain	[43]
scn9a	Voltage gated Na^+ channels	Chronic pain syndromes, experimental pain	[44]
Genes associated with the synthesis of interleukins			
il10	Interleukin-10	Chronic pain syndromes	[45, 46]
il1b	Interleukin-1β	Chronic pain syndromes	[47, 48]
il6	Interleukin-6	Chronic pain syndromes	[49]
Tnfa	Tumor necrosis factor, α	Chronic pain syndromes	[50, 51]

Genes associated with certain metabolic response			
mt1x	Metallothionein	Dependent conditions	52
vldlr	Very low density lipoprotein receptor	Dependsent conditions	52
znf-ld	Transcriptional regulator of DNA methylation	Chronic pain syndromes, experimental pain	53

After the analysis of the SNP polymorphisms annotations in the database NCBI-SNP is selected 10 genes with interconnection variants of identified phenotype variations with non-synonymous SNP-semantic polymorphisms. For such polymorphisms have been described real variants of the amino acid in one of the positions of the amino acid sequence of the encoded protein, leading to a change of the spatial molecule conformation with the possible impact on its function. A list of these polymorphisms is presented in Table. 2, in which the above-described SNP polymorphisms were identified as relevant.

Table 2: A list of key genetic polymorphisms associated with phenotypic characteristics of pain perception and reactive response of the human organism on it

Gene	SNPs в NCBI-SNP			
	Number of records	**Only actual records**	**The most likely candidates**	**Effects on the phenotype**
adrb2	795	16	rs1800888 rs35336948	Sensitivity to drugs, the incidence of pain syndromes and cardiovascular diseases
comt	3980	29	rs9265 s74745580	General pain sensitivity and pain tolerance
grm1	74658	75	rs362829 rs6923492 rs362962	General pain sensitivity and pain tolerance
htr2a	15514	36	rs6314 rs2274639	The subjective pain tolerance
oprm1	28996	47	rs1799971 rs497976	The subjective pain tolerance, tendency to addiction
slc6a4	18642	41	rs28914822 rs25531	General pain sensitivity and pain tolerance
cacna2d3	180000	237	rs 6777055 rs1851048	General pain sensitivity and pain tolerance
il1b	2527	22	rs1143627 rs2853550 rs1799916	Tissue and general reaction to injury
Tnfa	802	11	s267600955	Tissue and general reaction to injury
znp-ld	11	1	rs2562456	The general reaction to the pain, and subjective pain tolerance

The analysis of the influence degree of the conformational variability of amino acid chains in the formation of the secondary structure of proteins on their likely functional properties allows to select as a promising the next 6 SNP: rs1851048 and rs 6777055 in *cacna2d3* gene, encoding the voltage gated Ca^{2+} channels; rs2562456 *znf-ld* gene of zinc-containing transcriptional regulator of DNA methylation; rs6923492 and rs362962 in *grm1*gene of metabotropic glutamate receptor; and Rs6314 in *htr2a* gene, encoding serotonin receptor type 2A.

Functional significance of each SNP seems to be concluded in alteration of peculiarities, which the encoded protein could express mediating specific manifestations of reactivity. The single

substitution in its amino acid chain lets to changes in affinity and/or activity of these receptors (GRM1 and HTR2A), channels (CACNA2D3), and gene regulators (ZNF-LD). These SNP variants could be used to develop the molecular diagnostics, allowing a high degree of reliability to detect genetically determined component of the general non-specific reactivity of the organism in the non-invasive study.

Conclusion

Bioinformatics analysis based on the full capture of the synonymous information from specialized databases of open access has allowed revealing twenty three genes with annotated SNP-polymorphisms linked to the variability of the pain perception and response of the organism to nociception. Due to the effect on the functional properties of the encoded proteins, up to ten of them can provide phenotypically significant properties of the organism in the composition of the GNRL phenomenon. The result is an actual list of the six SNPs with potentially high phenotypic variability, suitable for the manufacture of test systems for the prediction of a genetically determined component of GNRL.

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Примечания:

1. Типологические особенности системного ответа организма животных с различным уровнем общей неспецифической реактивности организма на стрессорно-повреждающие воздействия / В.Б. Писарев, В.В. Новочадов, А.Б. Мулик и др. // Вестник Волгоградского государственного медицинского университета. 1998. 54(4). С. 10-12.

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