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FAST ATOM BOMBARDMENT MASS SPECTROMETRY (FAB) IN EVALUATION OF BIOSYNTHETIC PATHWAYS OF PURINE RIBONUCLEOSIDE [^{2}H]INOSINE

In this paper was demonstrated the possibility of the use of FAB mass spectrometry on an impulse mass spectrometer VG-70 SEQ ("Fisons VG Analytical", USA) for the study of biosynthetic pathways of ^{2}H-labeled purine ribonucleoside inosine secreted into the liquid culture (LC) by Gram-positive chemoheterotrophic bacterium *Bacillus subtilis* VKPM B-3157 when grown on heavy water (HW) medium with 2 % hydrolyzate of deuterated biomass of methylotrophic bacterium *Brevibacterium methylicum* VKPM B-5662 as a source of growth ^{2}H-labeled substrates. Isolation of ^{2}H-labeled inosine from the 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 % ethanol 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 molecule of biosynthetic [^{2}H]inosine by FAB mass spectrometry demonstrated the incorporation of 5 deuterium atoms into the molecule (the total level of deuterium enrichment — 62.5 atom. % ^{2}H) with incorporation of 3 deuterium atoms 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 reactions of enzymatic izomerization of glucose in ^2H_2O-medium due to reactions of glycolysis, associated with the Embden-Meyerhof pathway with participation of reactions of isotope (^{1}H–^{2}H) exchange, while two other deuterium atoms at C2, C8-positions in the hypoxanthine residue were synthesized from [^{2}H]amino acids that originated from the deuterated hydrolysate of the methylotrophic bacterium *Brevibacterium methylicum* VKPM B-5662.

Кл. сл.: : ^{2}H-labeled inosine, biosynthesis, heavy water, Bacillus subtilis VKPM B-3157, mass spectrometry FAB

INTRODUCTION

Natural nucleosides labeled with deuterium (^{2}H) are of considerable scientific and practical interest for various biochemical and diagnostic purposes [1], structural-functional studies [2], and are used in template-directed syntheses of molecules of deuterated RNA for studying their spatial structure and conformational changes [3]. The trends to a preferred use of stable isotopes as compared to their radioactive counterparts are stipulated by the lack of radioactive radiation hazards and the possibility of using the instrumental methods of mass spectrometry for determination of the localization of the deuterium label in molecules [4]. As an analytical method mass spectrometry has exceptionally high sensitivity and allows the detection and quantification of micro and trace amounts of organic substances in large volumes of liquids and gases, as well as in biological systems [5]. In the case of FAB fast atoms are formed by preionization in the discharge of noble gas atoms (Ar, Xe), acceleration of the ions and their subsequent neutralization in a collision with uncharged atoms [6]. FAB mass spectrometry is used for analyzing substances which are soluble or dispersible in the liquid matrix (glycerol, thioglycerol, polyethylene glycol, *m*-nitrobenzyl alcohol, etc.) [7]. FAB mass spectra different from the mass spectra of chemical ionisation in that they contain ion peaks $[M + H]^+$ or [M – H], but not $M^{+\cdot}$ or $M^{-\cdot}$. The FAB method can analyze the molecules of different nature with a molecular weight up to 10 kDa [8]. The FAB method is especially useful in the study of the compounds having acidic, basic or ionic groups. However, the FAB method is less sensitive than the electron ionization and matrix-assisted laser desorption, and has little use for quantification, but it is useful for the qualitative determination of non-volatile substances without chemical modification into the volatile derivatives.

The main obstacle for practical usage of deuterated nucleosides in the above-mentioned studies 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 0.015 atom. %, however, despite low deuterium content in specimens, recently developed methods for its enrichment and

purification allow to produce ^{2}H-labeled substrates with high isotopic purity.

Inosine (1,9-dihydro-9-beta-D-ribofuranosyl-6H-purin-6-one) — ribonucleoside consisting of hypoxanthine, bound to the ribose residue (ribofuranose) by means of β-N9-glycosidic bond, having a polyfunctional biological effects on the organism. In eukaryotic cells and protozoa inosine serves as a precursor of the ATP, enhances the activity of some enzymes of the Krebs cycle, stimulates the synthesis of nucleotides. As a metabolic remedy, inosine has antihypoxia, metabolic and antiarrhythmic effect, but also exerts a positive effect on metabolic processes in myocardium, improves coronary circulation, preventing the degenerative changes of the myocardium caused by physical exertion, infection or endocrine diseases [9].

For biosynthesis of natural ^{2}H-labeled nucleotides, including inosine have been developed approaches using as growth substrates the hydrolysates of deuterated biomass of methylotrophic bacteria, assimilating methanol *via* the ribulose-5'-monophosphate (RMP) and serine pathways of carbon assimilation, an interest to which is increasing due to intensive development of technology for chemical synthesis of methanol [10, 11]. The assimilation rate of methylotrophic biomass by prokaryotic and eukaryotic cells makes up 85–98 %, and their productivity calculated on the level of methanol bioconversion into cell components reaches 50 % [12]. As we have earlier reported, methylotrophic bacteria are convenient objects capable to grow on minimal salt media containing 2–4 % [^{2}H]methanol, whereon other bacteria are unable to reproduce, and may easily be adapted to maximal ^2H_2O concentrations [13]. However, the experiments discovered a bacteriostatic effect of ^2H_2O consisted in inhibition of vitally important cell functions of the bacterial producer cells [14]. That is why a number of applied items regarding the biosynthesis of natural deuterated nucleosides in ^2H_2O remain to be unstudied.

The great scientific and practical interest toward the usage of analytical methods of mass spectrometry to the study of biosynthetic pathways of deuterated natural compounds identified the objective of this work involved the study of the possibility of using FAB mass spectrometry to establish biosynthetic pathways of ^{2}H-labeled purine ribonucleoside inosine, synthesized by Gram-positive chemoheterotrophic bacterium *Bacillus subtilis* VKPM B-3157.

INSTRUMENTS AND MATERIALS

To prepare the growth media used ^2H_2O (99.9 atom. % ^{2}H), ^{2}HCl (95.6 atom. % ^{2}H), [^{2}H]methanol (98.5 atom. % ^{2}H) obtained from the Russian Scientific-technical Center "Isotope" (St. Petersburg, Russia). Inorganic salts were recrystallized preliminary in ^2H_2O; ^2H_2O was distilled over $KMnO_4$ with the subsequent control of isotope enrichment by ^{1}H-NMR-spectroscopy on a Brucker WM-250 device ("Brucker Daltonics", Germany) (working frequency — 70 MHz, internal standard — Me_4Si), proton chemical shifts (δ, ppm) were measured in parts per million relative to Me_4Si.

UV-spectrophotometry was registered with Beckman DU-6 programmed spectrophotometer ("Beckman Coulter", USA) within the wavelength range of λ = 220–280 nm.

Analytical determination of inosine was 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, vol. %. Spots were eluted with 0.1 N HCl. The UV absorbance of eluates was recorded spectrophotometrically at λ = 249 nm, using a standard calibration plot.

Ion exchange chromatography was performed on Biotronic LC 5001 apparatus ("Eppendorf-Nethleler-Hinz", Germany) using a column with Biotronic resin BIC 2710; t = 20–(±25) °C; column dimensions — 3.2 × 230 mm. The stationary phase — sulfonated styrene resin (7.25 % of cross-linking) UR-30 ("Beckman Spinco", USA); mobile phase — 0.2 M of Na-citrate buffer; operating pressure — 50–60 atm; feed rate of Na-citrate buffer — 18.5 ml/h; ninhydrin — 9.25 ml/h; detection at λ = 570 nm and λ = 440 nm (for proline).

The high performance liquid chromatography was carried out on a liquid chromatograph Knauer Smartline ("Knauer", Germany) equipped with a UV detector UF–2563 and integrator C–R 3A ("Shimadzu", Japan) at t = 20–(±25) °C using 250 × 10 mm column with the stationary phase of Ultrasorb CN, 10 μm ("Kova", Slovakia). The mobile phase — acetonitril–water = 75 : 25 vol. % under the gradient elution conditions; the sample volume — 50–100 μl; elution rate — 1.5 ml/min.

FAB mass spectrometry was carried out on an impulsed mass-spectrometer VG-70 SEQ ("Fisons VG Analytical", USA) equipped with a cesium source (Cs^+) on a glycerol matrix with accelerating voltage 8–30 kV and ion current 0.6–0.8 mA by pre-ionization of samples in the discharge of the noble gas atoms (Ar, Xe).

EXPERIMENTAL

Strain-producer of [^{2}H]inosine

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

Biosynthesis of [^{2}H]inosine

[^{2}H]inosine was produced by microbiological synthesis with an output 3.9 g/l in heavy water (HW) medium (89–90 atom. % ^{2}H) using 2 % hydrolysate of deuterated biomass of the methanol assimilating strain of the facultative Gram-positive methylotrophic bacterium *Brevibacterium methylicum* VKPM B-5662 as a source of ^{2}H-labeled growth substrates. The strain was obtained by multistage adaptation on a solid 2 % agarose mineral salt M9 medium and 2 % [^{2}H]methanol with a stepwise increasing gradient of ^2H_2O concentration (from 0 up to 98 vol. % ^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 %) was added into HW-medium with ^2H_2O (mass. %): glucose — 12.0, hydrolysate of deuterated biomass of *B. methylicum* VKPM B-5662 — 2.0, NH_4NO_3 — 2.0, $MgSO_4 \cdot 7H_2O$ — 1.0, $CaCO_3$ — 2.0, adenine — 0.01, uracil — 0.01. As a control was used equivalent protonated medium containing 2 % yeast protein–vitamin concentrate (PVC), which composition is similar to that of HW-medium except 2 % PVC of yeast. The cultivation was performed in 500 ml Erlenmeyer flasks (containing 100 ml of the growth medium) for 3–4 days at t = +32 °C under intensive aeration on an orbital shaker S-380 ("Biorad Labs", Hungary). The bacterial growth was controlled on the ability to form individual colonies on the surface of solid (2 % agarose) media, as well as on the optical density (OD) 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. The level of bioconversion of carbon substrate was determined by the method as described in [15].

Isolation of [^{2}H]inosine

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 at t = 0 °C (3 × 5 ml). The mixture was kept for ~10 h at t = +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 t = +4 °C. The water fraction was separated by filtration; the solid phase was supplemented with 20 ml 50 % ethanol solution in 25 % ammonia (1 : 1, vol. %) and heated at t = +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 % ethanol ($[\alpha]_D^{20}$ = +1.61°, output 3.1 g/l (80 %)). The samples were then applied to 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 t = –10 °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 (liquid matrix — glycerol; accelerating voltage Cs^+ — 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 %.

RESULTS AND DISCUSSION

Preparation of deutero-biomass of *B. methylicum* VKPM B-3157

For this study was used a mutant strain of the Gram-positive chemoheterotrophic bacterium *B. subtilis* VKPM 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), which due to impaired metabolic pathways involved in the regulation of the biosynthesis of

* Here in after are used the volume percentages

purine ribonucleosides, this strain under standard growth conditions (PVC-medium, late exponential growth, t = +32 °C) synthesizes 17–20 g of inosine per 1 liter of the LC [16]. The maximal yield of inosine was attained on a protonated medium containing as a source of carbon and energy glucose (12 mass. %), and as a source of growth factors and amine nitrogen 2 % of yeast protein–vitamin concentrate (PVC). During biosynthesis 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 the autoclaved biomass of the Gram-positive facultative methylotrophic bacterium *B. methylicum* VKPM B-5662, capable to assimilate methanol *via* ribulose-5'-monophosphate 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 % ^2H_2O and 2 % [^{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 [17]. The adaptation of *B. methylicum* VKPM 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 up to 98 % ^2H_2O) in the presence of 2 % methanol and its deuterated analog, because we assumed that gradual cell adaptation to ^2H_2O would have a favorable effect on the growth parameters of the strain (Table 1).

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* VKPM B-5662 was cultivated on protonated M9 medium with water and methanol, the duration of lag-period and cell generation time were 20.0 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.

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

Experiment number	Media components, % (v/v)				Lag-period, h	Yield of biomass, % from the control	Generation time, h
	H_2O	^2H_2O	Methanol	[^{2}H]methanol			
1	98.0	0	2	0	20.0	100.0	2.2
2	98.0	0	0	2	30.0	92.3	2.4
3	73.5	24.5	2	0	32.1	90.6	2.4
4	73.5	24.5	0	2	34.0	85.9	2.6
5	49.0	49.0	2	0	40.5	70.1	3.0
6	49.0	49.0	0	2	44.1	60.5	3.2
7	24.5	73.5	2	0	45.8	56.4	3.5
8	24.5	73.5	0	2	49.6	47.2	3.8
9	0	98.0	2	0	58.3	32.9	4.4
10	0	98.0	0	2	60.0	30.1	4.9
10'	0	98.0	0	2	40.0	87.0	2.8

* The data in experiments 1–10 are submitted for *B. methylicum* VKPM B-5662 at growing on growth media M9 with 2 % methanol/[^{2}H]methanol and specified amounts (vol. %) ^2H_2O. The data in Expt. 10' are submitted for adapted for maximum content of deuterium in growth medium bacterium at the growing on growth media with 2 % [^{2}H]methanol and 98 % ^2H_2O. As the control used experiment where used ordinary protonated water and methanol.

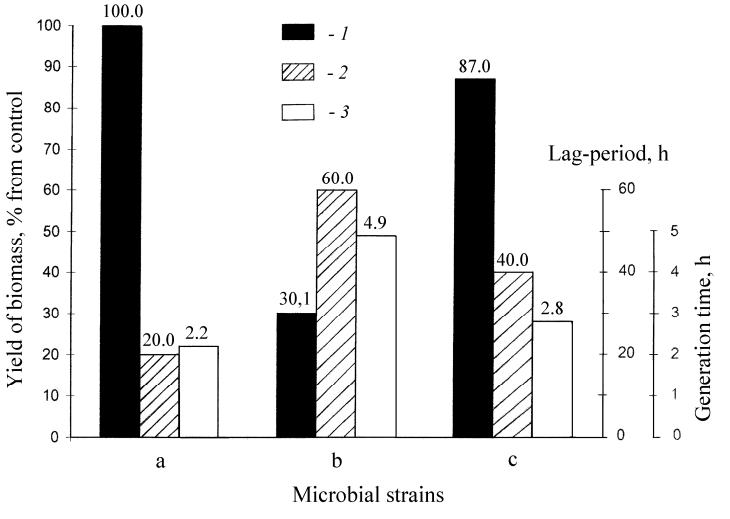

Fig. 1. Yield of microbial biomass of *B. methylicum* VKPM B-5662 (*1*), the magnitude of lag-period (*2*) and generation time (*3*) in various experimental conditions: a — initial strain on protonated medium (control) with water and methanol; b — initial strain on maximally deuterated medium; c — adapted to deuterium strain on maximally deuterated medium.

The most remarkable values of this parameters were detected in experiment 10, in which was used the maximally deuterated medium with 98 % ^2H_2O and 2 % [^{2}H]methanol; the lag-period and cell generation time in these conditions were increased in 3.0- 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 was monitored by recording the growth dynamics of the initial bacterium (b) and adapted to deuterium (c) bacterial strain (Fig. 1, the control (a) obtained in protonated medium), as well as by the change in the duration of the lag-period, time of generation and output of the microbial biomass. Unlike the adapted bacterium (c), the growth dynamics of the initial bacterium (b) on the maximally deuterated medium were inhibited by deuterium (Fig. 1). The yield of biomass for adapted methylotroph (c) was decreased approx. on 13 % in comparison with control conditions (a) at an increase in the time of generation up to 2.8 h and the lag-period up to 40 h (Fig. 1). 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 % ^2H_2O and 2 % [^{2}H]methanol, incubation period 3–4 days at t = = +32 °C.

Biosynthesis of [^{2}H]inosine

The strategy for the biosynthesis of [^{2}H]inosine with using the biomass of *B. methylicum* VKPM B-5662 as growth substrates was developed taking into account the ability of methylotrophic bacteria to synthesize large amounts of protein (output 50 % of dry weight), 15–17 % of polysaccharides, 10–12 % of lipids (mainly, phospholipids), and 18 % of ash [18]. 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 N ^{2}HCl (in ^2H_2O). Since the inosine-producing strain *B. subtilis* VKPM 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 % ^2H_2O and 2 % [^{2}H]methanol, and the levels of their deuterium enrichment (Table 2). Qualitative and quantitative composition of the amino acid from methylotrophic hydrolysate was studied on cation exchange column "Biotronic LC-5001" (Germany), with the sulfonated resin UR-30 and the levels of deuterium enrichment of molecules — by mass spectrometry EI methyl esters of N-5-(dimethylamino)naphthalene-1-sulfonyl (dansyl) amino acid derivatives as described in [19]. As it shown in 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 %, 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 medium and levels of deuterium enrichment (atom. %) of molecules

Amino acid	Yield, % dry weight per 1 gram of biomass	The value of the molecular ion of amino acid derivatives $[M]^{+*}$	Number of deuterium atoms incorporated into the carbon backbone of a molecule*	Level of deuterium enrichment of molecules, % of the total number of hydrogen atoms**
Glycine	9.69	324	2	90.0
Alanine	13.98	340	4	97.5
Valine	3.74	369	4	50.0
Leucine	7.33	383	5	49.0
Isoleucine	3.64	383	5	49.0
Phenylalanine	3.94	420	8	95.0
Tyrosine	1.82	669	7	92.8
Serine	4.90	355	3	86.6
Threonine	5.51	ND***	–	–
Methionine	2.25	ND	–	–
Asparagine	9.59	396	2	66.6
Glutamic acid	10.38	411	4	70.0
Lysine	3.98	632	5	58.9
Arginine	5.27	ND	–	–
Histidine	3.72	ND	–	–

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

** When calculating the level of deuterium enrichment protons (deuterons) in the COOH and NH_2-groups of amino acid molecules are not taken into account because of the ease of isotope (^{1}H–^{2}H) exchange.

*** ND — not detected.

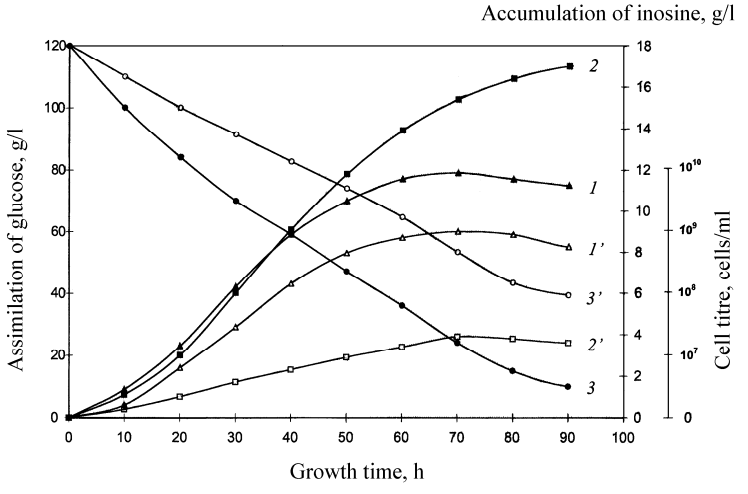

Fig. 2. Growth dynamics of *B. subtilis* VKPM B-3157, cells/ml (*1*, *1*'), inosine accumulation, g/l (*2*, *2*') and glucose assimilation, g/l (*3*, *3*') under different experimental conditions: *1, 2, 3* — protonated PVC-medium; *1, 2', 3* — HW-medium with 2 % hydrolysate of deuterated biomass of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

Table 3. Qualitative and quantitative compositions of intracellular carbohydrates isolated from *B. subtilis* VKPM 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 from PVC-medium (control)	Sample from the HW-medium	
Glucose	20.01	21.40	80.6
Fructose	6.12	6.82	85.5
Rhamnose	2.91	3.47	90.3
Arabinose	3.26	3.69	90.7
Maltose	15.30	11.62	–
Sucrose	8.62	ND**	–

* The data were obtained by ^{1}H NMR-spectroscopy.
** ND — not detected.

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 49.0 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* VKPM B-5662 as a source of growth substrates for growing the inosine-producing strain *B. subtilis* VKPM B-3157. *methylicum* VKPM B-5662 (Fig. 2). Experiments demonstrated a certain correlation between the changes in growth dynamics (Fig. 2, experiments *1*, *1*'), output of inosine (Fig. 2, experiments *2*, *2*'), and glucose assimilation (Fig. 2, experiments *3*, *3*'). The maximal output of inosine (17 g/l) was observed on the protonated PVC-medium at a glucose assimilation rate 10 g/l (Fig. 2, experiment *2*). The output of inosine on the HW-medium decreased in 4.4-fold, reaching 3.9 g/l (Fig. 2, experiment *2*'), and the level of glucose assimilation — 4-fold, as testified by the remaining 40 g/l non-assimilated glucose in the LC (Fig. 2, experiment *3*'). The experimental data demonstrate that glucose is less efficiently assimilated during the growth on the HW-medium as compared to the control conditions on the protonated PVC-medium owing due to inhibition of enzymatic reactions with heavy water the assimilation of glucose by the cell. 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* VKPM B-3157, which was performed by the HPLC method on an Ultrasorb CN column, 10 μm with a mixture of acetonitrile–water = 75 : 25, vol. % 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

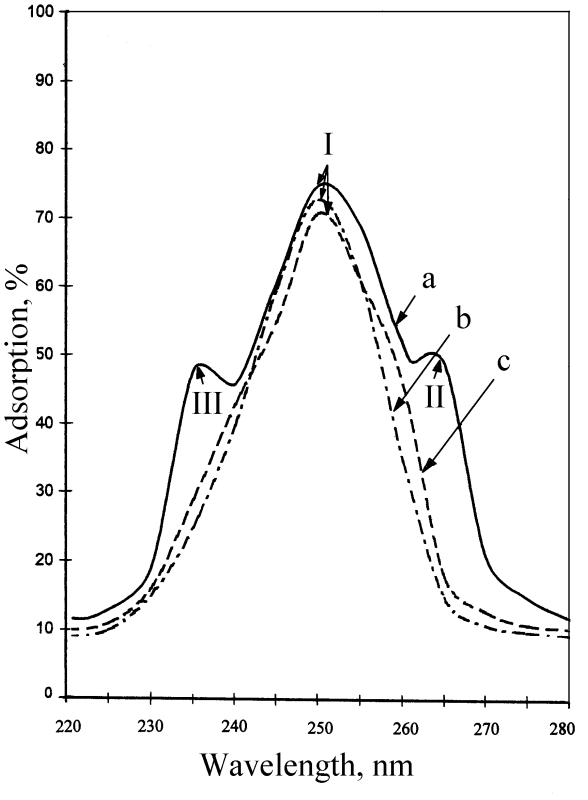

Fig. 3. UV-absorption spectra of inosine (0.1 NHCl): a — the initial LC after the growth of *B. subtilis* VKPM B-3157 on HW-medium; b — natural inosine; c — inosine isolated from the LC of the strain-produser. I — inosine, II and III — secondary metabolites

of 3.08 (15.63 %), 4.26 (7.46 %), 7.23 (11.72 %) and 9.14 (7.95 %) min (not shown). As was expected, the output of glucose in the deuterated hydrolysate was 21.4 % of dry weight, that is, higher than the outputs of fructose (6.82 %), rhamnose (3.47 %), arabinose (3.69 %), and maltose (11.62 %) (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.

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

The use of a combination of physical-chemical methods for isolating [^{2}H]inosine from the LC was determined by the need for preparing inosine of a high chromatographic purity (no less than 95 %). Since the 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), the LC was fractionated in a stepwise manner for isolating of [^{2}H]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 the 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 the 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 ethanol–ammonia solution at + 60 °C; inosine – by extracting with 0.3 M ammonium–formate buffer (pH = 8.9) and subsequent crystallization in 80 % 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 data on isolation of inosine from the LC of *B. subtilis* VKPM B-3157 at various stages of isolation procedure are presented in the form of UV-absorption spectra in Fig. 3, a, b, *c*. The presence of major absorbance band I, corresponding to natural inosine (λ_{max} = = 249 nm, ε_{249} = 7100 $M^{-1}\cdot cm^{-1}$), and the absence of secondary metabolites II and III in the analyzed sample (Fig. 3, c), demonstrates the homogeneity of isolated product and the efficiency of the isolation method.

The study of distribution of the deuterium label in the molecule of [^{2}H]inosine

The level of deuterium enrichment of the [^{2}H]inosine molecule was determined by FAB mass spectrometry, which due to ionization by fast atom allows detecting micro and trace amounts of organic substances in large volumes of fluids and biological systems without preliminary chemical modification into the volatile derivatives. For this aim, we recorded FAB mass spectra of protonated and deuterated inosine, by the difference of the peak values of molecular ions was performed the calculation of the level of deuterium enrichment of the molecule. The formation of a molecular ion peak for inosine in FAB mass spectrometry was accompanied by the migration of proton H^+ with formation of molecules with $[M + H]^+$. Biosynthetically ^2H-labeled inosine

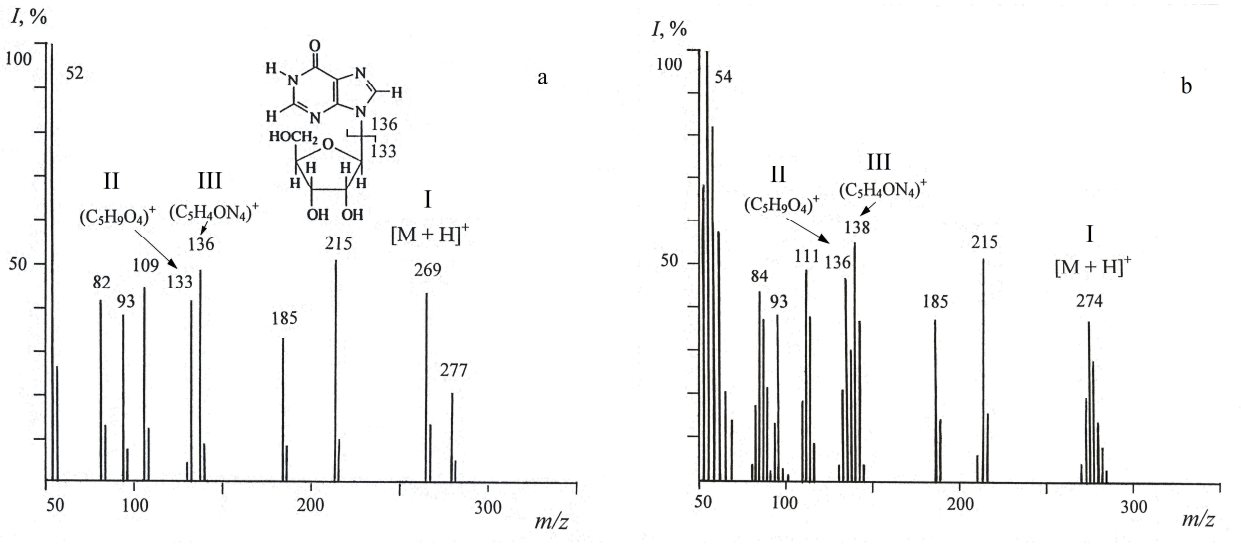

Fig. 4. FAB mass spectra of inosine (glycerol as a matrix) under different experimental conditions: a — natural inosine; b — [^{2}H]inosine isolated from HW-medium. Ionization conditions: cesium source under the discharge of Ar; accelerating voltage, 5–30 kV; ion current, 0.6–0.8 mA. I — inosine; II — ribose fragment; III — hypoxanthine fragment

Table 4. Values of peaks $[M + H]^+$ in the FAB mass spectra and levels of deuterium enrichment (atom. %) of the inosine molecule

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
274	38	5	62.5
275	28	6	72.5
276	14	7	87.5

* 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 $H_2O/^2H_2O$.

(the FAB mass-spectrum is represented in Fig. 4, b regarding the control (natural protonated inosine, Fig. 4, a), represented a mixture of isotope-substituted molecules with different numbers of hydrogen atoms replaced by deuterium. Correspondingly, the molecular ion peaks of inosine $[M + H]^+$ were 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, 42 % under the control conditions (Fig. 4, a), that result corresponds to 5 deuterium atoms incorporated into the inosine molecule (Fig. 4, b). Peak II with *m*/*z* = 133, 41 % and peak III with m/z = 136, 49 % correspond to the hypoxanthine fragment and the ribose fragment. As shown in Table 4, the molecular ion peak of inosine $[M + H]^+$ 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. This indicates the isotopic polymorphism at this instrumental method of introduction into the molecule of deuterium label.

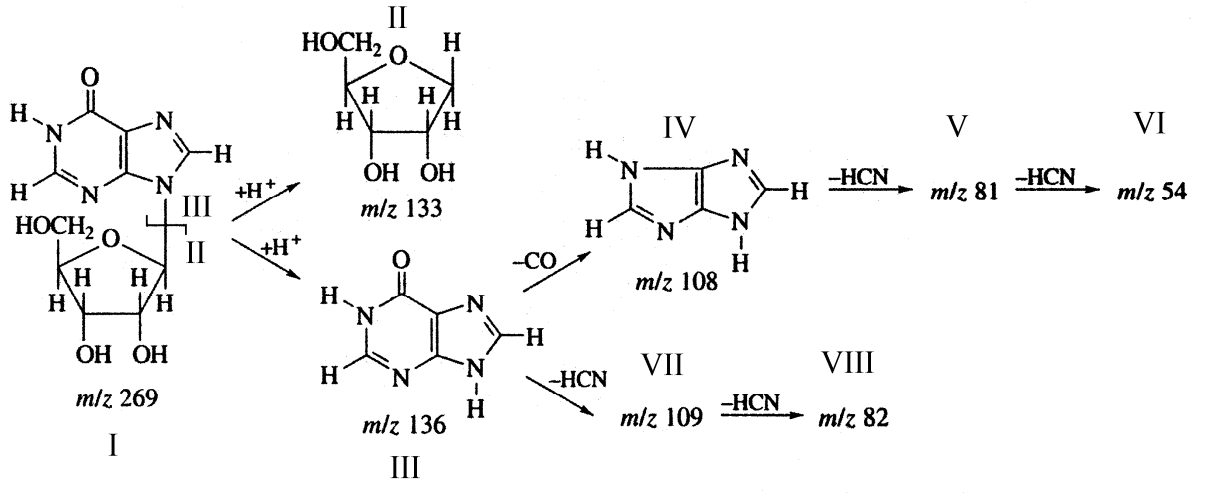

Fig. 5. The fragmentation pathways of the inosine molecule by the FAB-method

Taking into account the effect of isotopic polymorphism 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 62.5 atom. % of the total number of non-exchangeable hydrogen atoms in the carbon backbone of the molecule:

$$TLDE = \frac{[M_{r1}]C_1 + [M_{r2}]C_2 + \ldots + [M_{rn}]C_n}{\sum C_n},$$

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

The more precise information on the deuterium distribution in the molecule gives the fragmentation of the inosine molecule, shown in Fig. 5. The fragmentation pathways of the inosine molecule by the FAB method lead to formation of ribose fragment (II) at m/z = 133 and hypoxanthine 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 (IV–VIII) at m/z = 109, 108, 82, 81, and 54 due to HCN and CO elimination from hypoxanthine (Fig. 5).

Consequently, the presence of two "heavy" fragments of ribose II at m/z = 136, 46 % (instead of m/z = 133, 41 % in the control) and hypoxanthine III 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 (VII, VIII), 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. The 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.

When analyzing the level of deuterium enrichment of [^{2}H]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) by this strain of chemoheterotrophic bacteria. 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. The carbon source was glucose as a main substrate and a mixture of deuterated amino acids from deuterated hydrolysate of methylotrophic bacterium *B. methylicum* VKPM B-5662 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

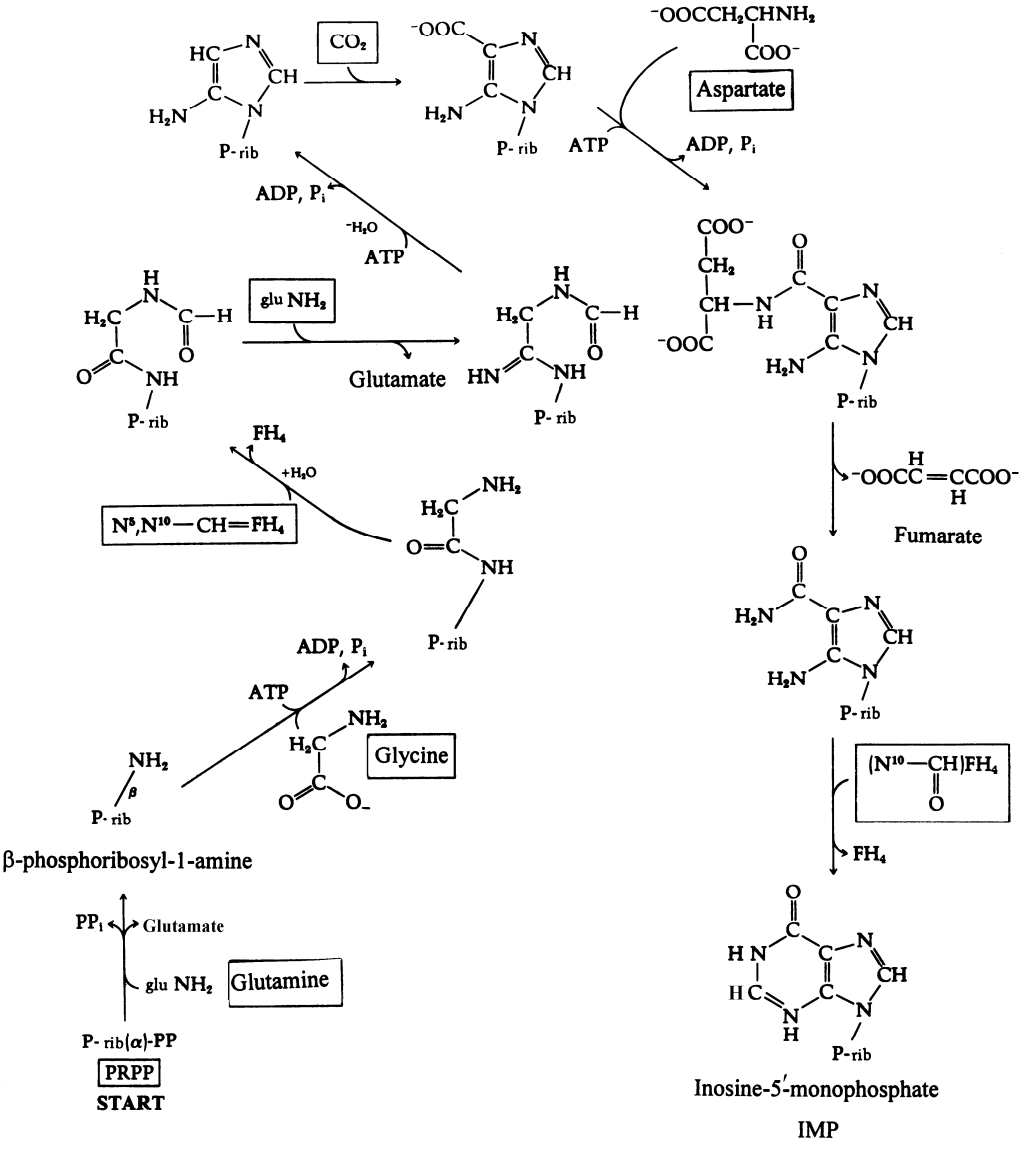

Fig. 6. Overall scheme of biosynthesis of IMP in microbial cell (adapted from [21])

residue is mainly determined by the assimilation of glucose by glycolysis, associated with the Embden—Meyerhof pathway. 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, contrary to this deuterium was incorporated into the ribose residue of the inosine molecule owing to reactions of enzymatic izomerization of glucose and isotopic ^1H–^2H exchange processes during the izomerization. These 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^-) protons protons (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 *via* enzymatic assimilation of glucose by the cell, while two other deuterium atoms at C2, C8-positions in the hypoxanthine residue could be synthesized at the expense of [^2H]amino acids as glycine, glutamine and aspartate (with participation of N^{10}–CHO–FH_4 and N^5,N^{10}–CH=FH_4) (Fig. 6), that originated from the

deuterated hydrolysate of methylotrophic bacterium *B. methylicum* VKPM B-5662. 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 [20]. However, it should be noted that auxotrophy of this mutant strain in tyrosine, histidine, adenine and uracil presupposes the branched metabolic pathways, different from those indicated above, since it is known that intermediates in glycolysis are precursors to a number of compounds as nucleotides and amino acids. The level of deuterium enrichment of the inosine molecule is determined by isotopic purity of ^2H_2O and deuterated substrates and, therefore, for the total administration of the deuterium label into the inosine molecule instead of protonated glucose it must be used its deuterated analogue.

CONCLUSION

According to the study, mass spectrometry FAB has worked well for the study of biosynthetic pathways of ^{2}H-labeled inosine and distribution of deuterium in the molecule. As a result, it was managed to precisely localize deuterium in the molecule of biosynthetic [^{2}H]inosine. The observed isotopic polymorphism, accompanied by proton migration to form $[M + H]^+$ is not an obstacle for determination of deuterium in the molecule, which is determined by taking into account the contribution of molecular ion peaks in the total level of deuteration molecule (5 atoms ^{2}H, 62.5 atom. %). From total 5 deuterium atoms in the inosine molecule, 3 deuterium atoms in the ribose residue of the inosine molecule were synthesized owing to reactions of enzymatic izomerization of glucose by the cell and reactions of isotopic (^{1}H–^{2}H) exchange, while two other deuterium atoms at C2, C8-positions in the hypoxanthine residue could be synthesized from [^{2}H]amino acids — glycine, glutamate and aspartate, that originated from deuterated hydrolysate of *B. methylicum* VKPM B-5662. In addition, the reactions of isotopic (^{1}H–^{2}H) exchange could also lead to the specific incorporation of deuterium into the molecule. To achieve higher levels of deuterium enrichment levels of the final product one need carefully control the isotopic composition of the growth medium and eliminate all sources of additional protons, including the use of deuterated glucose, which may be isolated in gram-scale quntities from deuterated biomass of the methylotrophic bacterium *B. methylicum* VKPM B-5662. In the future it is planned to prepare and identify by this developed method other deuterated natural nucleosides and their analogues.

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