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ELECTRON IMPACT MASS SPECTROMETRY IN ANALYSIS OF INTRODUCTION OF STABLE ISOTOPES OF DEUTERIUM AND CARBON-13 INTO AMINO ACID MOLECULES FROM BIOLOGICAL OBJECTS

The work demonstrates the possibility of using electron impact mass spectrometry (EI) on a MB-80A device ("Hitachi", Japan) with a double electron focusing for analysis of [^{2}H, ^{13}C]amino acid mixtures of *L*-phenylalanine producing strain of *Brevibacterium methylicum* and *L*-leucine-producing strain of *Methylobacillus flagellatum*, and [^{2}H, ^{13}C]amino acids of the total biomass protein isolated while growing the bacterial cells on media containing as a source of stable isotopes [^{2}H]methanol, [^{13}C] methanol and ^2H_2O. For mass-spectrometric analysis of the level of incorporation of stable isotopes ^{2}H and ^{13}C into the molecules of [^{2}H, ^{13}C]amino acids the multi-componential mixtures of [^{2}H, ^{13}C]amino acids, derived from cultural media and protein hydrolysates after hydrolysis in 6 M ^{2}HCl (3 % phenol) and 2 M $Ba(OH)_2$ were modified into N-benzyloxycarbonyl-derivatives of [^{2}H, ^{13}C]amino acids as well as into the methyl esters of N-dansyl-derivatives of [^{2}H, ^{13}C]amino acids, which were separated by the method of reverse phase high performance liquid chromatography (RP HPLC) on a column with octadecylsilane gel Separon SGX C18. The levels of ^{2}H and ^{13}C enrichment of secreted amino acids and amino acid residues of protein were found to be varied depending on the metabolic pathways of biosynthesis and concentration of ^{2}H- and ^{13}C-labelled substrates in growth media from 20.0 atom. % to *L*-leucine/isoleucine up to 97.5 atom. % for *L*-alanine.

Keywords: stable isotopes, methylotrophic bacteria, isotope labeled amino acids, RP HPLC, EI mass spectrometry

INTRODUCTION

The method of isotope labeling is an important instrumental tool for various biochemical and metabolic studies using amino acids and other biologically active substances (BAS), labeled with stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O) [1]. 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 determination of the localization of the label in the molecule by high resolution techniques, including NMR [2], IR-spectroscopy [3] and mass spectrometry [4]. The development of these methods for the detection of stable isotopes in recent years has significantly increased the efficiency of biological research, as well as studies of the structure and mechanism of action of cellular BAS at the molecular level. In particular, ^{2}H- and ^{13}C-labeled amino acids are used for studying of the spatial structure and conformational changes of proteins, the interaction of protein molecules and in chemical syntheses of some isotope-labeled compounds based on them [5, 6].

An important factor in studies with using [^{2}H, ^{13}C]amino acids is their accessibility. [^{2}H, ^{13}C]amino acids can be prepared using chemical, enzymatic and microbiological methods. Chemical synthesis is multistage often require a large expenditure of costly labeled reagents and substrates and leads to a racemic mixture of *D*- and *L*-enantiomers for the separation of which is required the special separation methods [7]. The fine chemical synthesis of ^{2}H- and ^{13}C-labeled amino acids are linked with using a combination of chemical and enzymatic approaches [8].

Microbiology provides an alternative to chemical synthesis a method for obtaining of amino acids labeled with stable isotopes, which leads to high yields of the synthesized products, to the effective incorporation of stable isotopes in the molecule, and to preservation of the natural configuration of synthesized [^{2}H, ^{13}C]compounds. When microbiological preparation of [^{2}H, ^{13}C]amino acids use several approaches, one of which is consisted in uniform enrichment of synthesized compounds by stable isotopes at the carbon skeleton in the molecule due to the using of bacterial strains growing on selective media containing as a source of stable isotopes [^{13}C]methanol, [^{2}H]methanol and ^2H_2O [9, 10]. This approach also involves the use of complex chemical components of biomass grown on [^{2}H, ^{13}C]growth substrates with further separating and fractionating of target ^{2}H- and ^{13}C- labeled compounds. Another approach consists in a site-specific enrichment of amino acids at certain positions of molecules due to assimilation by cell the isotopically labeled precursors such as

[1,4–^{13}C]succinate, [1, 2-^{13}C]acetate and [1-^{13}C]lactate [11].

The present work is a continuation of the research related to the usage of electron impact (EI) mass spectrometry in analyzing of the level of incorporation of stable isotopes of ^{2}H and ^{13}C into complex multicomponent mixtures of [^{2}H, ^{13}C]amino acids obtained microbiologically due to the utilization by microbial cells of low-molecular-labeled substrates — [^{2}H]methanol, [^{13}C]methanol and $^{2}H_2O$. The sensitivity of mass spectrometry is 10^{-9}–10^{-11} mol in samples, which is considerably higher than when using IR- and NMR spectroscopy. This method in combination with reverse phase high performance liquid chromatography (RP HPLC) has worked well for the study of the level of isotopic enrichment of [^{2}H, ^{13}C]amino acid molecules in the composition of their multi component mixtures as the samples of culture liquids of bacterial strains, producers of amino acids and hydrolysates of total protein of biomass obtained on minimal growth media containing isotopic labeled substrates.

INSTRUMENTS AND MATERIALS

For the synthesis of derivatives of [^{2}H, ^{13}C]amino acids used N-5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride), chem. pur. ≥99.0 % (HPLC), (Sigma Aldrich, USA), benzyloxycarbonyl (CBz-chloride), ≥98.0 % (HPLC) ("Sigma Aldrich", USA) and diazomethane, prepared from N-nitrosomethylurea, ≥99.0 % (HPLC) ("Sigma Aldrich", USA). To prepare the growth media used $^{2}H_2O$ (99.9 atom.% ^{2}H), ^{2}HCl (95.6 atom. % ^{2}H), [^{2}H]methanol (98.5 atom. % ^{2}H) and [^{13}C]methanol (99.5 atom. % ^{13}C) obtained from the Russian Scientific-technical Center "Isotope" (St. Petersburg, Russia). Inorganic salts were recrystallized preliminary in $^{2}H_2O$; $^{2}H_2O$ 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.

The separation of the mixture of methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids from the culture liquid and protein hydrolysates was carried out at t = 20–(±25) °C by the RP HPLC method on a liquid chromatograph Knauer Smartline ("Knauer", Germany) equipped with a UV detector UF-2563 and integrator-R 3A ("Shimadzu", Japan) using 250×10 mm column with the stationary phase of Separon SGX C18, 7 μm ("Kova", Slovakia). The mobile phase: (A) acetonitril— trifluoroacetic acid = 100: (0.1–0.5) vol. % and (B) acetonitrile = 100 vol. % under the gradient elution conditions. The sample volume — 50–100 μl; elution rate — 1.5 ml/min. The yield of methyl esters of the individual N-Dns-[^{2}H, ^{13}C]amino acids was 75–89 %; the chromatographic purity — 95–98 %.

Ion exchange chromatography of protein hydrolysates was performed on Biotronic LC 5001 apparatus ("Eppendorf-Nethleler-Hinz", Germany) using a column with Biotronic resin BIC 2710; t = 20–(±25) °C; 3.2×230 mm. The stationary phase: sulfonated styrene (7.25 % of cross-linking) resin 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; ninhydrin — 9.25 ml/h; detection at λ = 570 nm and λ = 440 nm (for proline).

Mass spectra of EI of amino acid derivatives were recorded on MB-80 A ("Hitachi", Japan) with a double focusing with ionizing voltage of 70 eV, an accelerating voltage of 8 kV and the temperature of the cathode source of 180–200 °C. The scanning of samples was analyzed at a resolution of 7500 arbitrary units using 10 % image sharpness.

EXPERIMENTAL

Strains-producers of [^{2}H, ^{13}C]amino acids

Investigations were carried out with genetically marked strains of bacteria obtained from the culture collection of the Russian National Collection of Industrial Microorganisms (VKPM) of State Research Institute of Genetics and Selection of Industrial Microorganisms:

1) *Brevibacterium methylicum VKPM B 5652* — *L*-leucine-dependent strain of facultative methylotrophic bacteria producing *L*-phenylalanine;

2) *Methylobacillus flagellatum KT* — *L*-isoleucine-dependent strain of obligate methylotrophic bacteria producing *L*-leucine.

Growing conditions and isolation of ^{2}H, ^{13}C-labeled proteins and amino acids

The cultivation of methylotrophic bacteria *B. methylicum* and *M. flagellatum* was performed in a mineral M9 medium in Erlenmeyer flasks with 250 ml volume filled up with 50 ml of the growth medium according to a method described in [12], using as a source of stable isotopes [^{2}H]methanol, [^{13}C]methanol and $^{2}H_2O$ in the presence of *L*-leucine for *B. methylicum* and *L*-isoleucine for *M. flagellatum* in concentrations of 10 mg/l. Cells were separated by centrifugation in a centrifuge T-24 ("Heraues Sepatech", Germany) (10000 g, 20 min). In the culture liquids were analyzed the secreted amino acids. To isolate the protein fraction of the total biomass the cells were washed twice with distilled water followed by centrifugation (10000 g, 20 min), exposed to ultrasound at 40 kHz (3×15 min) and centrifuged. The resulting precipitate (10 mg) obtained after the separation of

lipids and pigments by a mixture of organic solvents: chloroform—methanol—acetone (2 : 1 : 1) was used as the protein fraction of the total biomass. Lipids and pigments were extracted with chloroform–methanol–acetone (2 : 1 : 1) according to the method of E. Bligh and W. Dyer [13]. The hydrolysis of total protein was performed with 6 M ^{2}HCl (3 % phenol in ^2H_2O) or 2 M $Ba(OH)_2$ (110 °C, 24 h).

Synthesis of N-Dns-[^{2}H, ^{13}C] amino acids

For the synthesis of N-Dns-[^{2}H, ^{13}C]amino acids to 4–5 mg of lyophilized samples of culture liquid and protein hydrolysates dissolved in 1 ml of 2 M $NaHCO_3$, pH = 9–10 was added portionwise with stirring 25.5 mg of dansyl chloride in 2 ml of acetone. The reaction mixture was kept under stirring for 1 hour at t = 40 °C, then acidified with 2 M HCl to pH = 3.0 and extracted with ethyl acetate (3×5 ml). The combined extract was washed with water until pH = 7.0, dried over anhydrous sodium sulfate, the solvent was removed at 10 mm. Hg.

Synthesis of methyl esters of N-Dns-[^{2}H, ^{13}C] amino acids

Synthesis of methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids was carried out with using diazomethane. For obtaining of diazomethane to 20 ml of 40 % KOH dissolved in 40 ml of diethyl ether was added 3.0 g of wet nitrosomethylurea and stirred at ice-water bath for 15–20 min. After intensive gassing closure ether layer was separated, washed with ice water until pH = 7.0, dried over anhydrous sodium sulfate, and further used to treat N-[^{2}H, ^{13}C]-Dns-amino acids in composition of culture liquids and hydrolysates of total proteins of biomass.

Synthesis of N-Cbz-[^{2}H, ^{13}C]amino acids

For the synthesis of N-Cbz-[^{2}H, ^{13}C]amino acids to 1.5 ml cooled to 0 °C of culture liquid solution (50 mg) or protein hydrolyzate (5.4 mg) in 4 M NaOH were added in portions with stirring 2 ml of 4 M NaOH and 28.5 mg of benzyloxycarbonyl. The reaction mixture was kept at 0 °C, stirred for about 3 hours, acidified with 2 M HCl to pH = 3.0 and extracted with ethyl acetate (3×5 ml). The combined extract was washed with water until pH = 7.0, dried over anhydrous sodium sulfate, the solvent was removed at 10 mm. Hg.

RESULTS AND DISCUSSION

Isolation of [^{2}H, ^{13}C]amino acids from the culture liquids and protein hydrolysates

The objects of the study were the obtained by mutagenesis a *L*-phenylalanine-producing strain of the facultative methylotrophic bacteria *Brevibacterium methylicum*, assimilating methanol via ribulose-5-monophosphate cycle of carbon assimilation, and a *L*-leucine-producing strain of obligate methylotrophic bacteria *Methylobacillus flagellatum*, implements a 2-keto-3-deoxy-gluconate aldolase variant of ribulose-5-monophosphate cycle of carbon assimilation. To compensate the auxotrophy for *L*-leucine and *L*-isoleucine, these amino acids were added into the growth medium in the protonated form. The levels of accumulation of *L*-phenylalanine and *L*-leucine in the liquid medium of these strains-producers reached values of 0.8 and 1.0 g/l respectively [14, 15]. The inclusion of deuterium into the molecules of secreted amino acids and total proteins was carried out via the cultivation of the strain of *B. methylicum* on mineral M9 media with ^2H_2O and protonated methanol, as the level of inclusion of ^{2}H into the amino acid molecules due to assimilation of [^{2}H]methanol is negligible.

Since the cell assimilates hydrogen (deuterium) atoms from H_2O (^2H_2O) environment, we selected conditions of deuterium enrichment of amino acid molecules and proteins under a stepwise increase in concentration of ^2H_2O in growth media as is shown in Table 1. The growth of microorganisms on ^2H_2O containing growth media was characterized by increasing the duration of the lag phase, the cell generation time, and the reduction of outputs of the microbial biomass (Table 1), so it was necessary to carry out the adaptation of cells to ^2H_2O.

The method of the optional adaptation of the strain of methylotrophic bacteria *B. methylicum* to grow on ^2H_2O while maintaining the ability for the biosynthesis of *L*-phenylalanine was described in article [16]. In this research, were investigated the samples of the culture liquids and biomass hydrolysates obtained during the multi-stage adaptation of *B. methylicum* to heavy water on minimal mineral M9 media with different content of ^2H_2O (from 24.5 to 98.0 % ^2H_2O). Since this strain of methylotrophic bacteria was adapted to grow in ^2H_2O, the study of inclusion levels of deuterium into the amino acid molecules is the most interesting.

Unlike the bacterial growth on ^2H_2O medium, wherein it was necessary to carry out the cell adaptation to deuterium, while at the preparation of [^{13}C]amino acids via assimilation of $^{13}CH_3OH$ this stage was not required, because this isotopic substrate does not exert the adverse biostatic effect on the growth characteristics of methylotrophs (see Table 1). Therefore, in the case of the strain of obligate methylotrophic bacteria *M. flagellatum* the inclusion of ^{13}C into amino acid molecules was carried out in one step by growing the bacteria on water M9 media containing 1 % of [^{13}C]methanol as a source of carbon-13 isotope.

Table 1. The influence of the isotopic composition of growth media on the growth of strains of *B. methylicum* and *M. flagellatum*

Number of experiment	Growth media*	The value of lag-phase, h	The output of biomass, % from control	The generation time, h
1	0	24.0	100	2.2
2	24.5	32.1	90.6	2.4
3	49.0	40.5	70.1	3.0
4	73.5	45.8	56.4	3.5
5	98.0	60.5	32.9	4.4
6	CH_3OH	0	100	1.1
7	$^{13}CH_3OH$	0.1	72.0	1.0

* Data for experiments 1–5 were presented for *B. methylicum* while growing on aqueous M9 media containing 2 % methanol and a specified amount (vol. %) of ^2H_2O. Data for experiments 6–7 show for *M. flagellatum* when growing on aqueous M9 media containing 1 % methanol (6) or 1 % [^{13}C] methanol.

The main stages at the isolation of [^{2}H, ^{13}C]amino acids consist in following:

– growing the respective strains producers on growth media containing labeled substrates — [^{2}H]methanol, [^{13}C]methanol and ^2H_2O;

– isolation of culture liquids (CL) containing the secreted amino acids from microbial biomass;

– purification of lipids;

– cell disruption;

– isolation of fraction of total protein of biomass with their subsequent hydrolysis, derivatization of mixtures of amino acids by dansyl chloride, benzyloxycarbonyl chloride and diazomethane;

– separation of methyl esters of N-Dns-amino acid derivatives and N-Cbz-amino acid derivatives by RP HPLC;

– EI mass spectrometry of the obtained amino acid derivatives.

^{2}H and ^{13}C-labeled amino acids were isolated from lyophilized culture liquids of amino acid-producing strains of *B. methylicum* and *M. flagellatum*, and as part of the total protein hydrolysates of biomass. When isolating the total protein fraction it should be considered the presence of carbohydrates, lipids and pigments in samples. In this research was used the protein rich bacterial strains with a relatively low content of carbohydrates in them. The hydrolysis was subjected as a fraction of the total protein the residue obtained after the separation of exhaustive extraction of lipids and pigments by organic solvents (methanol—chloroform—acetone). In rare cases for the complete separation of the cellular components used the salting out by ammonium sulfate.

The hydrolysis of ^{2}H-labeled proteins was performed under conditions to prevent hydrogen isotopic exchange reactions with deuterium during the hydrolysis and preservation of the aromatic [^{2}H]amino acid residues in the protein. It was considered two alternatives variants of hydrolysis — the acid and alkaline hydrolysis. Acid hydrolysis of the protein under standard conditions (6 M HCl, 24 h, 110 °C), is known to induce the complete degradation of tryptophan and partial degradation of serine, threonine, and several other amino acids in the protein [17]. Another significant drawback when carrying out the hydrolysis in HCl consists in the isotopic (^{1}H—^{2}H) exchange of aromatic protons (deuterons) in molecules of tryptophan, tyrosine and histidine, as well as protons (deuterons) at the C3 atom of aspartic and C4 glutamic acids [18]. Therefore, to obtain the information about the real inclusion of deuterium into biosynthetically synthesized molecules of amino acids it was necessary to carry out the protein hydrolysis using deuterated reagents (6 M ^{2}HCl with 3 % phenol (in ^2H_2O)).

Another variant of hydrolysis of the protein was consisted in using 2 M $Ba(OH)_2$ (110 °C, 24 h). Under these conditions the reactions of isotopic (^{1}H—^{2}H) exchange at aromatic [^{2}H]amino acids — tyrosine and tryptophan do not occur, and tryptophan is not destroyed. Both these methods of hydrolysis showed good results for the conservation of aromatic [^{2}H]amino acids in the protein hydrolysate and the content of deuterium in molecules of [^{2}H]amino acids. However, for the preparative production of ^{2}H-labeled amino acids from the microbial protein is advisable to use the hydrolysis in ^{2}HCl in ^2H_2O (in the presence of phenol to maintain the aromatic amino acid) to prevent racemization.

For preparation of volatile derivatives the amino acids were converted into the methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids or N-Cbz-[^{2}H, ^{13}C]amino acids,

which were further separated by the RP HPLC method. The conditions of N-derivatization of [^{2}H, ^{13}C]amino acids were practiced so as to obtain as much as possible intensive molecular ion peaks (M^+) in EI mass spectra at the background level of the growth media metabolites. For this it was carried out the direct N-derivatization of [^{2}H, ^{13}C]amino acids in the composition of lyophilized culture liquids and total protein hydrolysates of biomass by 5-fold excess of dansyl chloride (in acetone) or benzyloxycarbonyl.

Under conditions of the reaction of N-derivatization for lysine, histidine, tyrosine, serine, threonine and cysteine along with mono-derivatives were formed N-di-Dns and N-di-Cbz-derivatives. In addition, from arginine was synthesized N-three-Dns-(Cbz)-arginine. Therefore, in mass-spectrometric studies the molecular ions (M^+) of these compounds were corresponded to di- or tri-derivatives.

The effectiveness of the use of N-Cbz- and N-Dns-amino acid derivatives in the RP HPLC and mass spectrometric studies was demonstrated by us previously [19]. The volatility of N-derivatives of amino acids in mass spectrometric analysis can be further enhanced by the esterification of the carboxyl group, so that N-Dns-[^{2}H, ^{13}C]amino acids were converted into their methyl esters. To prevent the reverse isotopic exchange of aromatic protons (deuterons) in the esterification of deuterium-labeled amino acids, in this paper we gave a preference to diazomethane. The freshly prepared solution of diazomethane in diethyl ether was treated with dry mixtures of amino acids residues. While the derivatization of amino acids with diazomethane it was occurred additional N-methylation at α-NH-(Dns)-group in [^{2}H]amino acids, leading to the appearance in the mass spectra of methyl esters of N-Dns-amino acids the additional peaks corresponding to compounds with a molecular mass on 14 mass units larger than the original compounds.

Study of levels of inclusion of stable isotopes of ^{2}H and ^{13}C into molecules of secreted amino acids and hydrolysates

The levels of inclusion of stable isotopes of ^{2}H and ^{13}C into the multicomponent mixtures of amino acid molecules of culture liquids and protein hydrolysates were determined analytically by the EI mass spectrometry method. Methyl esters of N-Dns-[^{2}H, ^{13}C]amino acid derivatives or N-Cbz-[^{2}H, ^{13}C]amino acid derivatives were separated by the RP-HPLC method on octadecylsilane gel Separon SGX C18, 7 μm. The best result on separation was achieved by the gradient elution of the methyl esters of N-Dns-[^{2}H, ^{13}C]amino acid derivatives with a mixture of solvents: (A): acetonitrile—trifluoroacetic acid = 100 : (0.1–0.5) vol. % and (B): acetonitrile 100 vol. % in the gradient elution conditions by gradually increasing the concentration of component B in the mixture from 0 to 100 % (see Table 2). In this case, each component of the mixture was separated in the most optimal composition of the eluent, thereby achieving their full separation quality in much less time than in isocratic mode. In addition, using the gradient significantly increased the maximum number of peaks in the chromatogram can accommodate — a peak capacity, which is

Table 2. The results of one-step gradient separation of a mixture of methyl esters of N-Dns-[^{2}H, ^{13}C]amino acids from hydrolyzates by the RP HPLC, t = 20–(±25) °C on a 250×10 mm column with octadecylsilane gel Separon SGX C18, 7 μ ("Kova", Slovakia)

Number of processing	Components of the mobile phase, vol.%		Elution time, min
	A*	B**	
1	90	10	10
2	80	20	10
3	60	40	10
5	50	50	10
6	30	60	5
8	20	80	5
9	10	90	5
10	0	100	5

*A : acetonitrile—trifluoroacetic acid = 100 : (0.1–0.5) vol. %
**B: acetonitrile = 100 vol. %

very important in the separation of complex multicomponent mixtures, which are the protein hydrolysates. Thus, it was possible to isolate tryptophan and intractable pair of phenylalanine/tyrosine. The chromatographic purity degrees of [^{2}H, ^{13}C]amino acids, isolated from culture liquids of *B. methylicum* and *M. flagellatum* and protein hydrolysates in the form of their N-Cbz-[^{2}H, ^{13}C]amino acid derivatives were comprised 96–98 % with yields — 67–89 %. For some [^{2}H, ^{13}C]amino acids was proved to be more convenient the separation as methyl esters of N-Dns-[^{2}H, ^{13}C] amino acid derivatives. The degrees of chromatographic purity of methyl esters of N-Dns-[^{2}H]phenylalanine, N-Dns-[^{2}H]tyrosine and N-Dns-[^{2}H]tryptophan make up 96, 97 and 98 %, respectively. This result is important because of the chemical stability of methyl esters of N-Dns-amino acids, the presence of high-molecular ions (M^+) at higher molecular weights have proved very convenient for mass spectrometric investigations and enable to identify [^{2}H,^{13}C]amino acids in the presence of low molecular weight metabolites of growth media and other products of derivatization. The latter fact is very important to study the composition of the pool of [^{2}H, ^{13}C]amino acids secreted into the culture liquids (CL) of corresponding strains-producers.

The fragmentation pathways of methyl esters of N-Dns-phenylalanine and N-Dns-leucine in the electron impact mass spectrometry lead to the formation of molecular ion peaks (M^+) at *m/z* = 412 and *m/z* = 378 and to the formation of dansyl fragments and products of the further decay to N-dimethylaminonaphthalene, and the formation of the amine fragment A^+ and aminoacyl fragment B^+ (Fig. 1). The fragmentation of methyl esters of N-Dns-phenylalanine and N-Dns-leucine shown on Fig. 1 is typical for these derivatives of all other amino acids, which allows to carry out the mass spectrometric monitoring of [^{2}H, ^{13}C]amino acids in the culture liquids of intact producing strains containing the amount of amino acids and other metabolites of the growth medium until the stage of chromatographic separation, as well as explore the inclusion of stable isotopes of ^{2}H- and ^{13}C into molecules of amino acids of protein hydrolysates.

When using as a source of stable isotopes [^{13}C]methanol and ^2H_2O in the cell are synthesized the isotopically-substituted [^{13}C, ^{2}H]amino acids differ in the number of atoms substituted on ^{13}C and ^{2}H. At the same time, the higher is the molecular weight of the amino acids, the larger is a set of possible molecular ions (M^+) corresponding to isotope-substituted forms. Peaks at *m/z* = 323.2, 337.4, 368.5, 382.3, 420.5 in the EI mass spectrum of [^{13}C]amino acid derivatives in the derivatized culture liquid of *M. flagellatum*, obtained in an aqueous medium with 1 % [^{13}C] methanol (Fig. 2, b) correspond by the weight to the methyl esters of N-Dns-[^{13}C]glycine, N-Dns-[^{13}C]alanine, N-Dns-[^{13}C]valine, N-Dns-[^{13}C]leucine/[^{13}C]isoleucine and N-Dns-[^{13}C] phenylalanine. It should be emphasized that the value of *m/z* for the molecular ion (M^+) of methyl esters of N-Dns-[^{13}C]leucine and [^{13}C]isoleucine in EI mass spectra is the same, so these amino acids could not be accurately identified by this method. The maximum levels of inclusion of ^{13}C isotope into amino acid molecules as measured by an increase of the averaged values of mass to charge ratio *m/z* for molecular ions (M^+) of isotopically-labeled sample in comparison with a molecular weight of a non-labeled natural amino acid are varied from 35 % for [^{13}C]alanine and 95 % for [^{13}C] phenylalanine (Fig. 2). Considering the auxotrophy of this strain for *L*-isoleucine, the variations in the range can be explained by the contribution of an exogenous isoleucine in the level of isotopic incorporation of [^{13}C]leucine, and other metabolically related amino acids – [^{13}C]alanine and [^{13}C]valine.

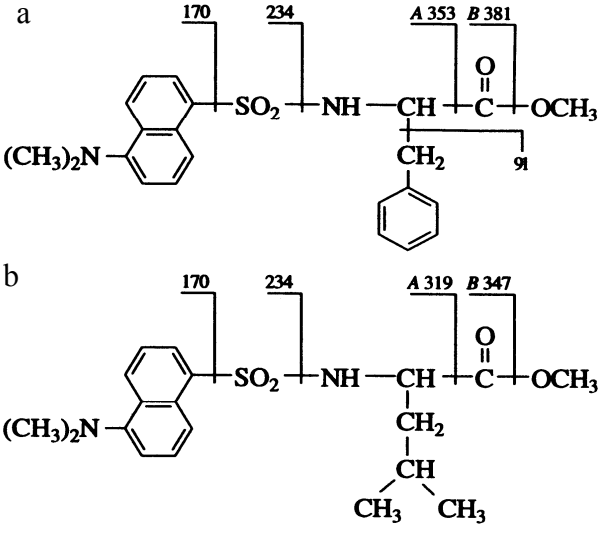


Fig. 1. Fragmentation of methyl esters of N-Dns-phenylalanine with M_r = 412 (a) and N-Dns-leucine M_r = 378 (b) in electron impact mass spectrometry

The mass spectrometry data on levels of inclusion of ^{13}C and ^{2}H into the molecule of N-Cbz-[^{2}H, ^{13}C]amino acid derivatives within various concentrations of ^2H_2O were not different from those of the methyl esters of N-Dns-[^{2}H, ^{13}C]amino acid derivatives (the accuracy of determination the levels of isotopic incorporation into amino acid molecules by this method comprises ± 5 %). As an example, Figure 3, b illustrates the mass spectrum of N-Cbz-[^{13}C]-Leu (relative to the unlabeled N-Cbz-Leu, Fig. 3, a) isolated by the RP HPLC method from LC of *M. flagellatum*

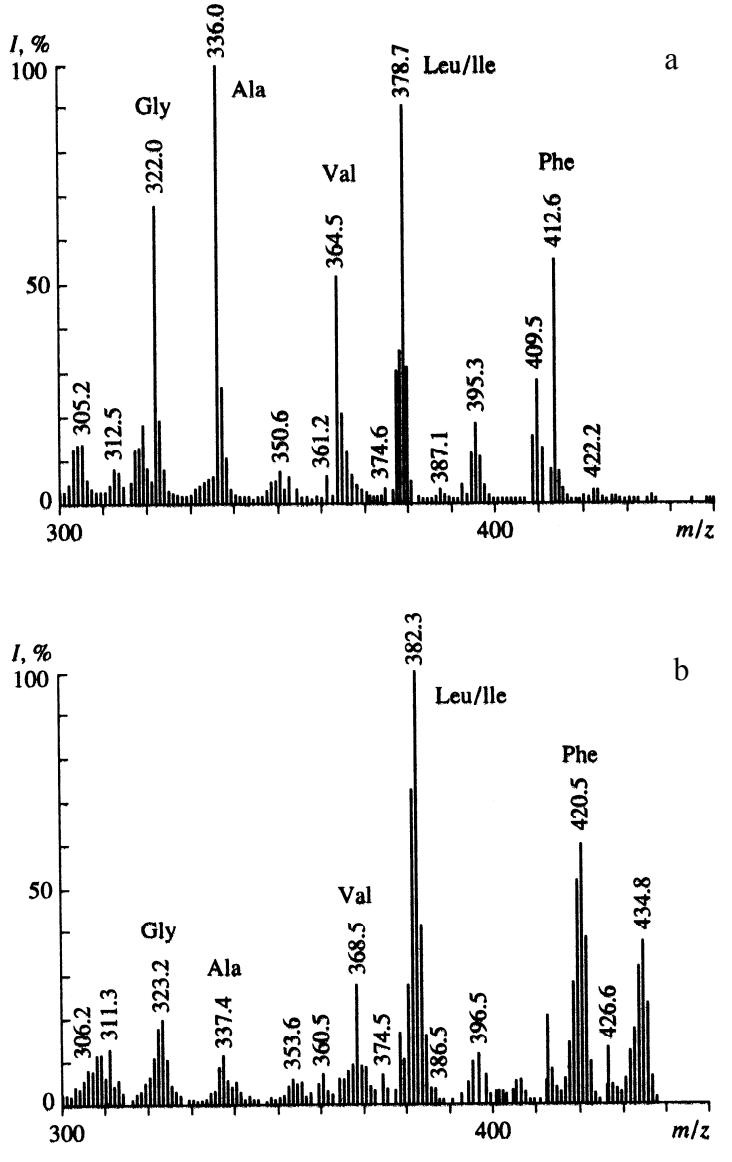
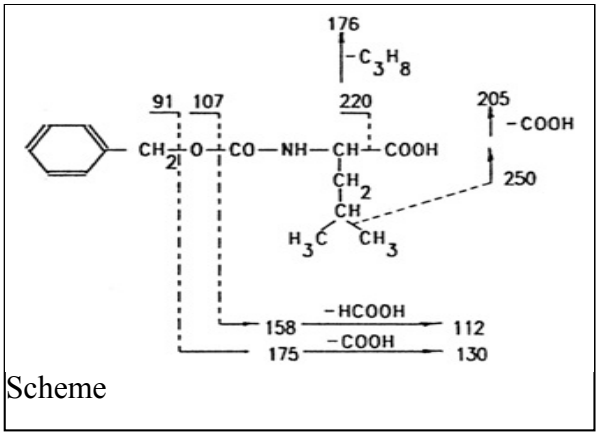


Fig. 2. EI mass spectra of methyl esters of N-Dns-[^{13}C]amino acids from LC of *M. flagellatum* after treatment with diazomethane and dansyl chloride.
a — 1 % methanol and H_2O (control);
b — 1 % [^{13}C] methanol and H_2O.
Symbols of amino acids are marked peaks of molecular ions $[M]^+$ of methyl esters of N-Dns-[^{13}C] amino acids. The intensity of the peaks is given in %

after the treatment by benzyloxycarbonyl. The peak of molecular ion of N-Cbz- [^{13}C]-Leu corresponds the mean value of (M^+) at *m/z* = 269 (relative to the (M^+) at *m/z* = 265 under the control conditions), indicating that the inclusion of 4 atoms of isotope ^{13}C into the molecule of leucine (Fig. 3, Scheme). The specific fragmentation of N-benzyloxycarbonyl derivative of [^{13}C]leucine under electron impact mass spectrometry makes it possible to localize the sites of incorporating of atoms of the isotope ^{13}C at the carbon skeleton of the molecule. As can be seen from Fig. 3, these are 4 carbon atoms in the positions [2, 3, 4, 5] of the carbon skeleton of the leucine molecule.

For the strain of methylotrophic bacteria *B. methylicum* there was a specific increase in the levels of isotopic incorporation of deuterium into molecules of individual [^{2}H]amino acids in the composition of culture liquids (Table 3) with stepwise increasing concentrations of ^2H_2O in growth media. The inclusion levels of deuterium into molecules of different [^{2}H]amino acids under the same growing conditions are varied. In all experiments was observed the proportional increase in the levels of isotopic incorporation of ^{2}H into the molecules of metabolically related [^{2}H]amino acids with stepwise increasing concentrations of heavy water in the growth media (Table 3). This result was recorded in all experiments where as a source of stable isotopes used ^2H_2O.



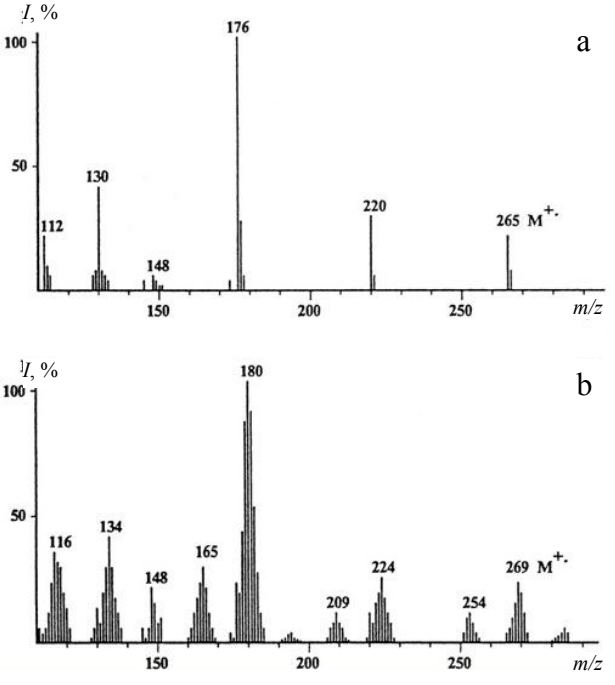


Fig. 3. EI mass spectra of N-Cbz-[^{13}C]-Leu, isolated from *M. flagellatum* after the treatment by benzyloxycarbonyl.
a — 1 % methanol and H_2O (the control); b — 1 % [^{13}C] methanol and H_2O.
The Scheme — the carbon skeleton of the molecule

Table 3. Levels of ^{13}C and ^{2}H inclusion into molecules of amino acids (atom. %), secreted into the culture liquid (CL) of *B. methylicum* and *M. flagellatum*, and into amino acid residues of protein

Amino acids	Content of ^2H_2O in the growth medium, %*								1% $^{13}CH_3OH$**	
	24.5		49.0		73.5		98.0			
	CL	Protein	CL	Protein	CL	Protein	CL	Protein	CL	Protein
Glycine	–	15.0	–	35.0	–	50.0	–	90.0	60.0	90.0
Alanine	24.5	20.0	50.0	45.0	50.0	62.5	55.0	97.5	35.0	95.0
Valine	20.0	15.0	50.0	46.0	50.0	50.0	55.8	50.0	50.0	50.0
Leucine /Isoleucine	20.0	15.0	50.0	42.0	50.0	50.0	50.0	50.0	40.0	49.0
Phenylalanine	15.0	24.5	27.5	37.5	51.2	50.0	75.0	95.0	95.0	80.5
Tyrosine	–	20.0	–	25.6	–	68.5	–	92.8	–	53.5
Serine	–	15.0	–	36.7	–	47.6	–	86.6	–	73.3
Aspartic acid	–	20.0	–	36.7	–	60.0	–	66.6	–	33.3
Glutamic acid	–	20.0	–	40.0	–	53.4	–	70.0	–	40.0
Lysine	–	10.0	–	35.3	–	40.0	–	58.9	–	54.4

* Data are submitted for inclusion of ^{2}H into the amino acid molecules when growing of *B. methylicum* on aqueous M9 media containing 2 % methanol and a specified amount (vol. %) of ^2H_2O.
** Data are submitted for inclusion of ^{13}C when growing of *M. flagellatum* on aqueous M9 media containing 1 % [^{13}C]methanol.

From the EI mass spectrum of methyl esters of N-Dns-[^{2}H]amino asid derivatives of culture liquid of *B. methylicum*, obtained on the growth medium containing 49 % ^2H_2O (Fig. 4, b) is shown that the phenylalanine molecule contains 6 isotopically-substituted forms with an average peak of molecular ion (M^+) with *m/z* = 414.2, which increases compared to the control conditions (*m/z* = 412.0, Fig. 4, a) on 2.2 units, i.e. 27.5 atom. % of the total number of hydrogen atoms in the molecule are substituted with deuterium. The region in the mass spectrum with values *m/z* = 90–300 corresponds to relevant products of derivatization of metabolites in the growth medium. The peak with *m/z* = 431.0, recorded in the EI mass spectrum of the culture liquid manifested in all the experiments, corresponds to the product of additional methylation of the phenylalanine molecule at α-NH-(Dns)-group. The peak with *m/z* = 400 (Fig. 4, b) corresponds to the product of cleavage of deuterated methyl group from the [^{2}H]phenylalanine derivative.

The presence in the EI mass spectrum of a sample of the culture liquid of *B. methylicum*, obtained on a medium containing 73.5 % ^2H_2O (Fig. 5) the molecular ion peak of the methyl ester of N-Dns-[^{2}H]phenylalanine (M^+) with *m/z* = 416.1 indicates on an increase in molecular weight of the [^{2}H]phenylalanine molecule on 4.1 unit i.e., 51.2 % of hydrogen atoms in the molecule of [^{2}H]phenylalanine in this case are replaced by deuterium. Evidently that above mentioned deuterium atoms were entered into the [^{2}H]phenylalanine molecule through biosynthesis *de novo*, i.e. to the carbon skeleton of the molecule. The protons (deuterons) at heteroatoms in the NH_2- and COOH-groups of amino acids are appertained to easily exchangeable ones, which are replaced by deuterium at the expense of ease of the dissociation in H_2O (^2H_2O).

From Table 3 it is shown that in conditions of auxotrophy in *L*-leucine the levels of inclusion of ^{2}H into the molecules of [^{2}H]leucine/[^{2}H]isoleucine are lower than those ones for phenylalanine. This feature more clearly manifests in the medium with the highest concentration of ^2H_2O. Once again, this result is confirmed in Figure 6, which shows the EI mass spectrum of methyl esters of N-Dns-[^{2}H]amino acids of culture liquid after the growth of the bacteria *B. methylicum* under these conditions.

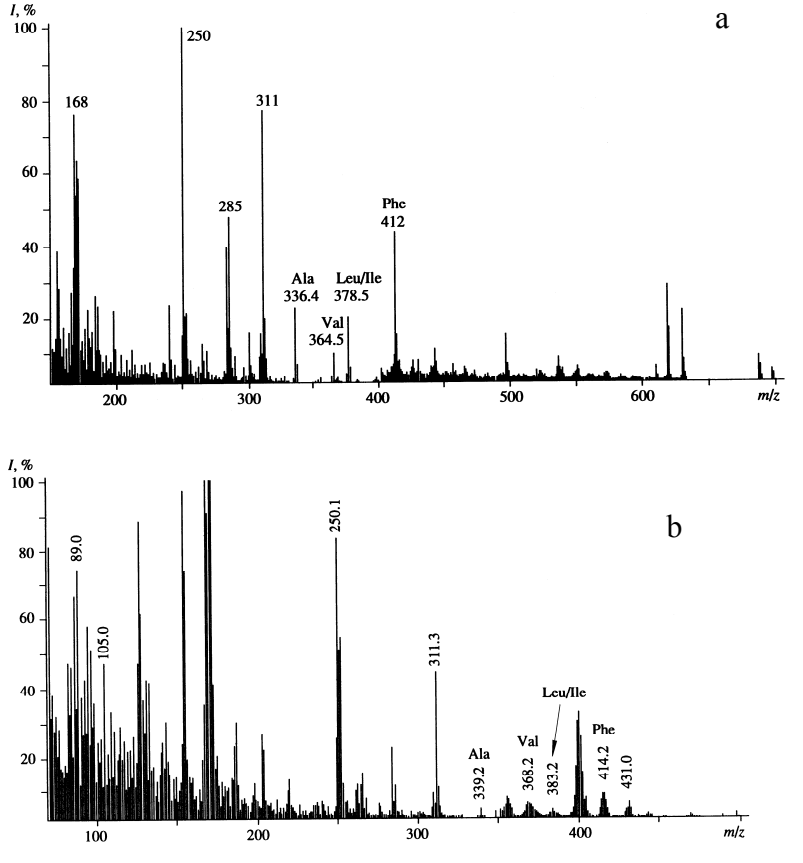


Fig. 4. EI mass spectrum of methyl esters of N-Dns-[^{2}H]amino acids from CL of *B. methylicum* after the treatment with diazomethane and dansyl chloride.
a — 2 % methanol and 98.0 % H_2O (control); b — 2 % [^{2}H]methanol and 49.0 % of ^2H_2O

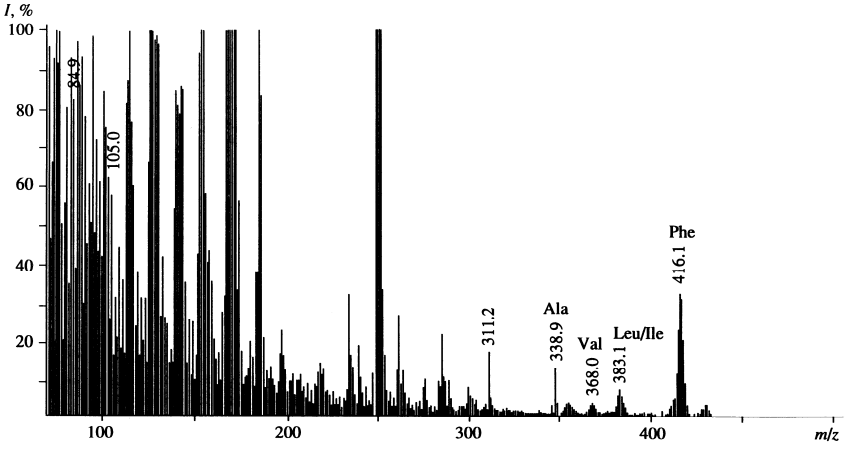


Fig. 5. EI mass spectrum of methyl ester of N-Dns-[^{2}H]amino acids from CL of *B. methylicum* when grown on growth medium containing 2 % [^{2}H]methanol and 73.5 % ^2H_2O

Clearly, the molecular ion peak of methyl ester of N-Dns-[^{2}H]phenylalanyl (M^+) with *m/z* = 418.0 increases compared to control conditions for 6 units corresponding to the substitution of 75.0 atom. % of the total number of hydrogen atoms in the molecule. Unlike [^{2}H]phenylalanine the inclusion level of deuterium enrichment in [^{2}H]leucine/[^{2}H]isoleucine was 50.0 atom. %, and [^{2}H]valine — 58.8 atom. %. The peak with *m/z* = 432, recorded in the EI mass spectrum of methyl esters of N-Dns-[^{2}H]amino acids of the CL in Fig. 6, corresponds to additional methylation product of [^{2}H]phenylalanine at α-NH_2-group. In addition, in the EI mass spectrum is recorded the peak enriched with deuterium the benzyl fragment $C_6H_5CH_2$ of [^{2}H]phenylalanine with *m/z* = 97 (instead of *m/z* = 91 in the control), indicating that the sites of localization of six deuterium atoms in the molecule of [^{2}H] phenylalanine are position of C1–C6 aromatic protons in the benzyl $C_6H_5CH_2$ fragment. From mass spectrometry data is demonstrated that at other concentrations of ^2H_2O in growth media deuterium is also included in the aromatic ring of [^{2}H]phenylalanine since the metabolism of the strain of *B. methylicum* adapted to ^2H_2O does not undergo significant changes in ^2H_2O.

A similar pattern in increasing levels of inclusion of ^{13}C isotope into amino acid molecules associated with auxotrophic metabolism was manifested when growing *L*-isoleucine-dependent strain of *M. flagellatum* on growth medium with 1 % [^{13}C] methanol. As can be seen from Table 3, unlike that observed for [^{13}C]phenylalanine (the level of isotopic incorporation— 95.0 %), the level of incorporation of ^{13}C isotope into the molecule of [^{13}C]leucine/[^{13}C]isoleucine, [^{13}C]alanine and [^{13}C]valine were 38.0, 35.0 and 50.0 % respectively. The level of isotopic incorporation into [^{13}C]glycine (60 %), was although higher than that for the last three amino acids, but significantly lower than that of [^{13}C]phenylalanine.

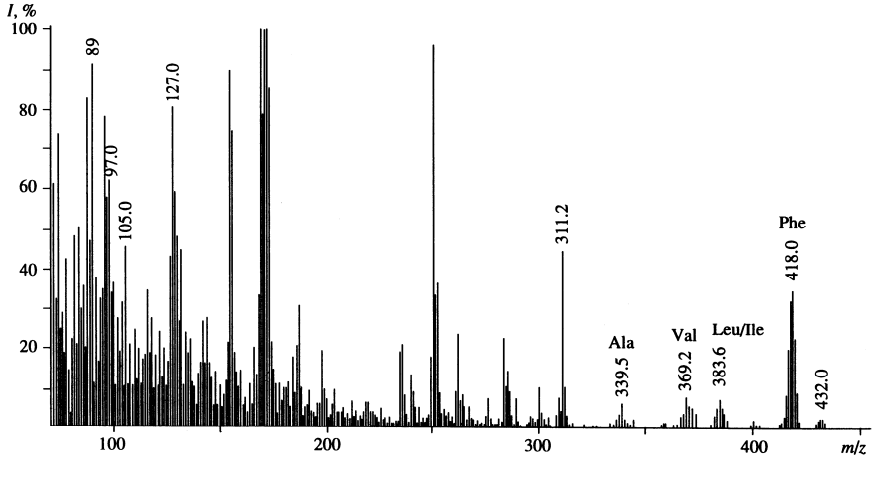


Fig. 6. EI mass spectrum of methyl esters N-Dns-[^{2}H]amino acids from CL of *B. methylicum* when growing on the growth medium containing 2 % [^{2}H]methanol and 98.0 % ^2H_2O (the maximum deuterated medium)

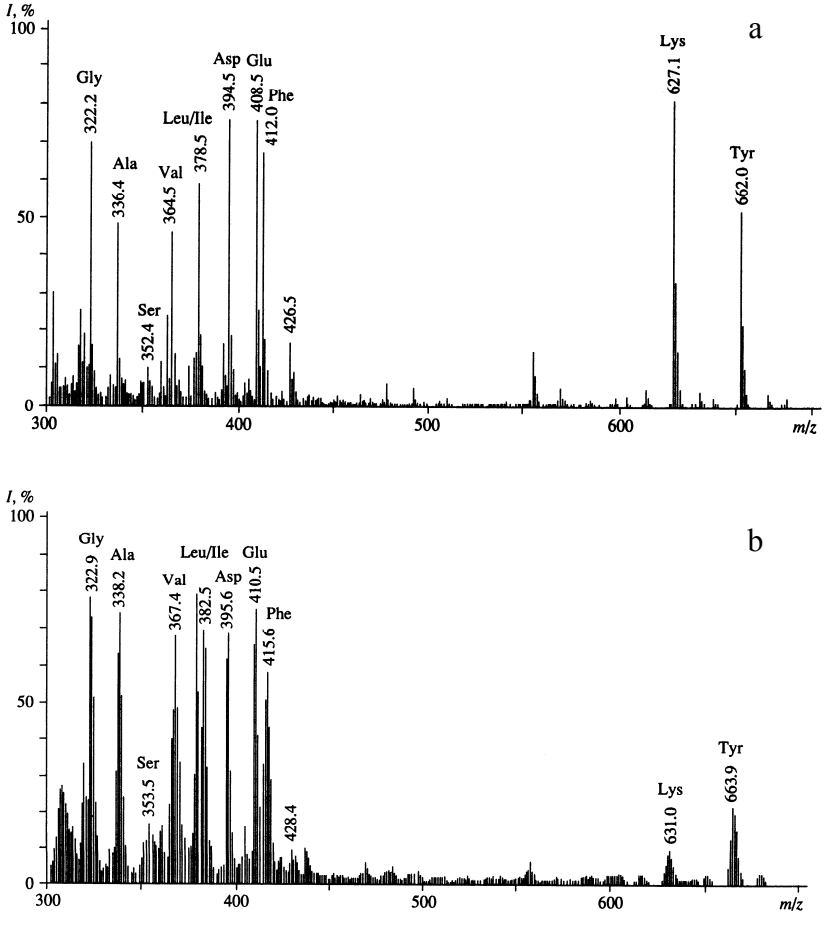


Fig. 7. EI Mass spectrum of methyl ester of N-Dns-[^{2}H]amino acids from hydrolyzates of total protein of biomass of *B. methylicum* when growning on M9 medium, containing 2 % methanol and H_2O (control) (a) and 2 % [^{2}H]methanol and 98.0 % ^2H_2O (b)

Summarizing data on the level of incorporation of ^{13}C and ^{2}H isotopes into secreted molecules of amino acids, it can be concluded about the maintaining of minor metabolic pathways associated with the biosynthesis of leucine and the metabolically related amino acids *de novo*. Another logical explanation for the observed effect, if we take into account the origin of leucine and isoleucine due to biosynthesis in various pathways (leucine belongs to the family of pyruvate, while isoleucine – to the family of aspartate), could be assimilation by the cell of the unlabeled leucine from the growth media under the background biosynthesis of isotopic-labeled isoleucine *de novo*. Taking into account these effects it should be emphasized that the use of auxotrophic forms of microorganisms for production isotope-labeled amino acids could not be justified practically because of the multiple character of inclusion of isotopes into the molecule [20]. On the contrary, the use for this purpose the prototrophic forms of microrganisms seem to be more promising for these aims.

The general principles for the study of levels of isotope inclusion of molecules of amino acids in this method of labeling were exemplified by the mass spectrometry analysis of complex multicomponent mixtures, obtained after total hydrolysis of proteins of biomass of methylotrophic bacteria *B. methylicum.* As seen in Figure 7, up to 10 amino acids may be identified in the protein hydrolyzate of *B. methylicum* by the peaks of molecular ions (M^+) of corresponding methyl esters of N-Dns-[^{2}H]amino acid derivatives.

As in the case with secreted amino acids, molecular ion peaks (M^+) were corresponded to isotopic mixtures of amino acid derivatives of substituted forms. For lysine and tyrosine peaks (M^+) were corresponded to di-methyl esters of amino acid derivatives — α, ε-di-Dns-lysine (($M^{+\cdot}$) at *m/z* = 631.0) and O,N-di-Dns-tyrosine ((M^+) at *m/z* = 663.9). The levels of isotopic incorporation of deuterium into the molecules of [^{2}H]amino acids from the hydrolysate of total protein biomass at the ^2H_2O content in the growth medium from 49.0 % to 25.6 % were varied from 25.6 % for [^{2}H]tyrosine to 45.0 % for [^{2}H]alanine (Fig. 8, b and Table 4). The levels of isotopic incorporation of deuterium into the molecules of [^{2}H]glycine, [^{2}H]valine, [^{2}H]phenylalanine, [^{2}H]serine, [^{2}H]lysine, [^{2}H]aspartic

and [^{2}H]glutamic acid are raged within 35–46 %. As in the case with secreted amino acids, with the increase of ^2H_2O concentration in growth media, it was observed the proportional increase in the level of incorporation of ^{2}H isotope into amino acid molecules. With regard to other [^{2}H]amino acids not detectable by this method, it is obvious that levels of isotope inclusion into the amino acid molecules are roughly the same. This is confirmed by data of separation of protein hydrolysates of methylotrophic bacteria by the RP HPLC method as N-Cbz-[^{2}H]amino acid derivatives and methyl esters of N-Dns-[^{2}H]amino acid derivatives and ion-exchange chromatography of protein hydrolysates, wherein it is detected 15 amino acids (Fig. 8, Table 4).

The findings suggest about the possibility of achieving maximum levels of inclusion of stable isotopes ^{2}H and ^{13}C into the amino acid residues of the total protein biomass (except for alanine, valine and leucine/isoleucine, reduced levels of inclusion of which explains the effect of auxotrophy for *L*-leucine and *L*-isoleucine). For example, in the case of the deuterated amino acid substitution at full stable isotopes has been achieved by using as a source of deuterium 98.0 % ^2H_2O (Table 4). As can be seen from Table 4, when growning of *B. methylicum* on growth medium with 98.0 % ^2H_2O, the inclusion levels of ^{2}H into residues of glycine, alanine, phenylalanine and tyrosine constitute 90.0, 97.5, 95.0 and 92.8 atom. %. In experiments on the inclusion of ^{13}C isotope into the total protein biomass due to the assimilation of [^{13}C]methanol by methylotrophic bacteria *M. flagellatum* were also observed high levels of isotopic incorporation in [^{13}C]glycine (90.0 %), [^{13}C]alanine (95.0 %) and [^{13}C]phenylalanine (80.5 %) (Table 3). As in the case with secreted amino acids the reduced inclusion levels of stable isotopes in [^{13}C]leucine/isoleucine (49.0 %), as well as related metabolic [^{13}C]amino acids under these conditions could be explained by the effect of auxotrophy of the strain in *L*-isoleucine, which was added into the growth medium in the protonated form.

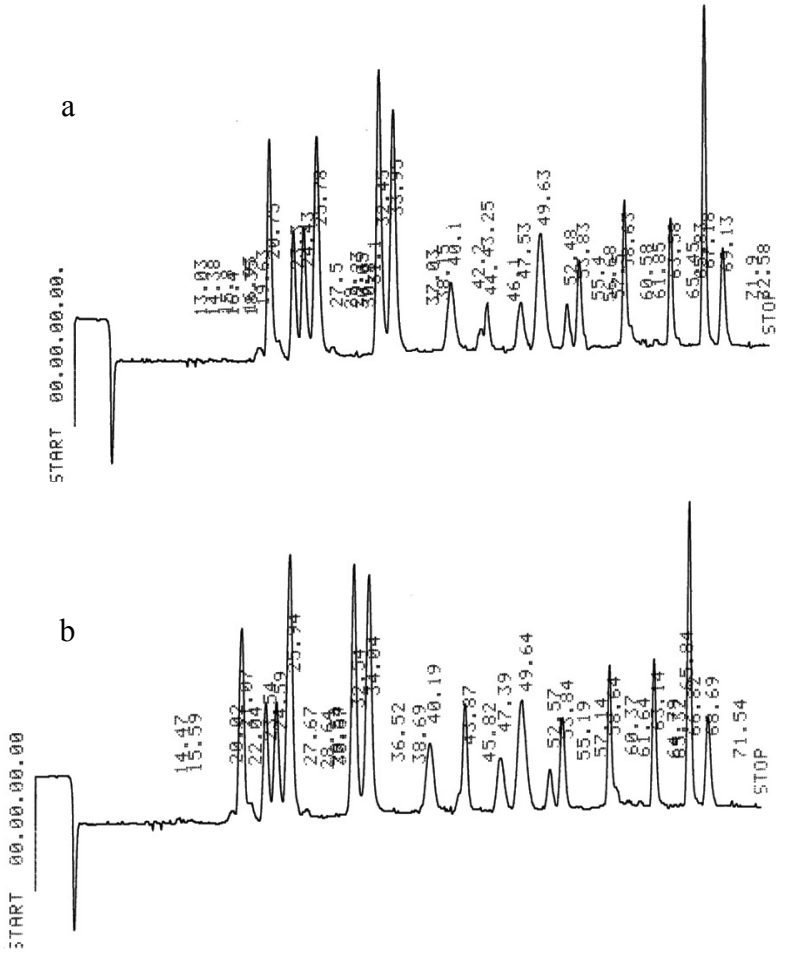


Fig. 8. Ion exchange chromatography of amino acids from hydrolysates of protonated (a) and deuterated (b) cells of *B. methylicum* on maximum deuterated M9 medium.
Biotronic LC-5001 (230×3.2 mm) ("Eppendorf-Nethleler-Hinz", Germany). The mobile phase: UR-30 sulfonated styrene resin ("Beckman-Spinco", USA). The pellet diameter — 25 mm; working pressure — 50–60 atm; mobile phase — 0.2 M Na-citrate buffer (pH = 2,5); eluent flow rate — 18.5 ml/h; ninhydrin — 9.25 ml/h; detection at λ = 570 and λ = 440 nm (for proline)

Table 4. The amino acid composition of a protein hydrolyzate of total protein of biomass of *B. methylicum*, obtained in the maximum deuterated growth medium* and levels of deuterium enrichment of molecules**

Amino acid	Output, % of dry weight of biomass		The number of included deuterium atoms in carbon skeleton of the molecule	The deuterium enrichment level, % from a total number of hydrogen atoms***
	Protonated sample (control)	Deuterated sample obtained in 98.0 % ^2H_2O		
Glycine	8.03	9.69	2	90.0
Alanine	12.95	13.98	4	97.5
Valine	3.54	3.74	4	50.0
Leucine	8.62	7.33	5	50.0
Isoleucine	4.14	3.64	5	50.0
Phenylalanine	3.88	3.94	8	95.0
Tyrosine	1.56	1.83	7	92.8
Serine	4.18	4.90	3	86.6
Threonine	4.81	5.51	–	–
Methionine	4.94	2.25	–	–
Aspartic acid	7.88	9.59	2	66.6
Glumatic 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	–	–

*Data obtained on M9 medium with 98.0 % ^2H_2O and 2 % [^{2}H]methanol.
**When calculating the deuterium enrichment level protons (deuterons) at COOH and NH_2 groups of amino acid molecules are not taken into account because of the ease of their dissociation and isotopic exchange in $H_2O/^2H_2O$.
***A dash indicates no data.

In all isotopic experiments on the integration of stable isotopes into the amino acid molecules the levels of inclusion of ^{2}H and ^{13}C into metabolically related amino acids found a certain correlation. Thus, the isotopic incorporation levels for alanine, valine and leucine (pyruvate family), phenylalanine and tyrosine (aromatic amino acid family, synthesized from shikimic acid) are correlated. At the same time levels of isotope inclusion for alanine, valine and leucine/isoleucine are stable within a wide variation of ^2H_2O concentration due to the effect of auxotrophy on leucine. The levels of isotopic incorporation for glycine and serine (serine family), aspartic acid, and lysine (asparagines family) also have similar values and are in correlation. The levels of isotopic incorporation into secreted amino acids and corresponding amino acid residues in the total protein when growing of bacteria on growth media with the same isotope content generally are well correlated. The reason for some of the observed differences in the level of inclusion of isotopes into amino acid molecules can be associated with the effect of auxotrophy of the used strains in leucine and isoleucine.

CONCLUSION

The research has demonstrated the effectiveness of the EI mass spectrometry method on the example of N-Cbz- and N-Dns-derivatives of [^{2}H, ^{13}C]amino acids with various levels of isotopic enrichment obtained by microbiological synthesis, to investigate the levels of isotopic enrichment of molecules of [^{2}H, ^{13}C]amino acids in composition of multicomponent mixtures produced microbiologically using microbial cells. The method is useful for studying the composition of the pool of amino acids, secreted into the culture liquid of strain-producers and protein hydrolysates of biomass, grown on media with stable isotopes of ^{2}H and ^{13}C, and can find further use in biomedical and metabolic studies.

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