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^{2}H- and ^{13}C-labeled amino acids generated by obligate methylotrophs Biosynthesis and MS monitoring

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Summary. Biosynthetic preparation of ^{2}H- and ^{13}C- labeled amino acids was studied using a leucine-producing mutant of the obligate methylotroph, *Methylobacillus flagellatum*. The strain was cultivated in various media containing ^{13}C- or ^{2}H-analogs of methanol. The total protein from each experiment was subjected to acid hydrolysis and converted into a mixture of dansyl amino acid methyl esters. The samples of excreted leucine were converted into methyl esters of dansyl and benzyloxycarbonyl derivatives. Electron impact mass spectrometry was performed to detect stable isotope enrichment of the amino acids. According to the mass spectrometric analysis it is feasible to use methylotrophic microorganisms for the preparation of ^{2}H- and ^{13}C- analogs of amino acids by labeled methanol bioconversion; the excreted amino acids can be convenient for express analysis as an indicator of isotopic enrichment of the total protein. The data obtained testified to a high efficiency of dansyl derivatization for mass spectrometric analysis of complex amino acid mixtures.

Keywords: Amino acids – Stable isotope labeled amino acids – Biosynthesis – *Methylobacillus flagellatum* – Electron impact mass spectrometry

Introduction

Stable isotope labeled amino acids are becoming an indispensable tool for protein structure research (Fesik and Zuiderweg, 1990; McIntosh and Dahlquist, 1990; Wuthrich, 1990) and the investigation of various aspects of metabolism (Ford et al., 1985; Loy et al., 1990). Direct chemical synthesis (Rohm and Van Etten, 1985; Van den Berg et al., 1988) still plays a central role in the preparation of amino acids labeled with stable isotopes. On the other hand the chemical route has significant limitations, it is very laborious and costly (Griffey and

Redfield, 1987; LeMaster, 1990). For many purposes the biosynthetic approach, which can include site-specific labeling (Kahana et al., 1988) and uniform isotopic enrichment (Crespi et al., 1968), promises to offer labeled amino acids and proteins in the quantities required for intensive NMR analysis and metabolic studies. The most appropriate sources of labeled biologically active compounds are microalgae and other microorganisms grown on culture media containing desirable labeled substrates (Lapidot and Kahana, 1986; Cox et al., 1988). The strategy for preparation of amino acids labeled with stable isotopes has been reviewed by LeMaster (1989; 1990) in respect to their use in NMR structural analysis of proteins. Beginning with the deuterium labeling experiments with algae (Crespi et al., 1968; 1970) up to the present, mainly two aspects of isotopic enrichment have been developed: novel expression systems and modern methods of bioanalysis of labeled compounds.

The biotechnological potential of methylotrophic microorganisms to produce various biologically active compounds utilizing C_1-metabolites is widely accepted (Linton and Niekus, 1987). Obligate methylotrophs are very attractive for biochemical research because these microorganisms can assimilate methanol as the sole source of carbon and energy. Stepwise oxidation of methanol via formaldehyde and formate to CO_2 competes with assimilation of formaldehyde via the 2-keto-3-deoxy-6-phosphogluconate aldolase/transaldolase (KDPGA/TA) variant of the ribulose monophosposphate cycle of methanol assimilation, resulting in cell material (Antony 1982; Southgate and Goodwin, 1989). To date the mechanism of formaldehyde assimilation has not been studied in detail.

We investigated the feasibility of using some amino acid-producing mutants of the obligate methylotroph *M. flagellatum* (Govorukhina et al., 1987; Kletsova et al., 1988) for the bioconversion of labeled methanol into excreted ^{2}H- and ^{13}C-amino acids and total cell protein.

For the routine analysis of numerous samples of excreted amino acids and total protein hydrolyzates from various *M. flagellatum* mutants grown on isotopically labeled media, a rapid and accurate method to determine isotopic enrichment was required. Electron impact mass spectrometry (MS) was used to monitor derivatized amino acids excreted into the culture media and total protein hydrolyzates isotopically enriched to different extents.

Materials and methods

Chemicals

$C^2H_3O^2H$ (99.8% atoms enriched with ^{2}H) was purchased from Aldrich Chem. Co (USA). ^2H_2O (99.9% atoms enriched with ^{2}H) was from All-Union Venture "Isotope" (Leningrad). $^{13}CH_3OH$ (96.0% atoms enriched with ^{13}C) was obtained from the Institute of Stable Isotopes (Tbilisi). Benzyloxycarbonyl chloride was purchased from the Protein Research Foundation (Japan). Dansyl chloride of sequential grade was from the Pierce Chem. Co (USA). Diazomethane was obtained from N-nitroso-N-methylurea from Sigma Chem. Co (USA). All other chemicals were of reagent grade.

The bacterial strains and growth conditions

The leucine-producing mutant of *M. flagellatum* was employed. Parental strain, *M. flagellatum KT*, was used as control of bacteria growth, other than excreted amino acids it did

not differ significantly from the leucine-producing mutant. Minimal salt medium (Miller, 1976) with methanol (1%, v/v) was used for bacteria growing. After autoclaving (at 120°C for 30 min) the basal medium was kept at room temperature, pH was adjusted till 7.4 with 4 N KOH and methanol was added as substrate. For the isotopic experiments the basal medium was supplemented with $^{13}CH_3OH$ or $C^2H_3O^2H/^2H_2O$ of various content (see Table 1 below). The stock cultures of *M. flagellatum* were maintained on plates using the same medium in the liquid culture with 15 g/l agar. The colonies, grown at 37°C for 3–4 days, were stored at 4°C. Cell biomass was cultivated in Erlenmeyer flasks filled to one-tenth their volume at 42°C and vigorously aerated on an orbital shaker for 68–72 h (cell density about 3.0 at 570 nm). The cells were harvested by centrifugation (280 g, 5 min). The supernatant was separated, lyophilized and the dry material was subjected to derivatization.

Protein hydrolysis

The bacterial cells were suspended in water and collected by centrifugation (280 g, 5 min). The washing procedure was repeated twice. The cells (35–40 mg) suspended in water (5 ml) were destroyed by sonication at 40 kHz for 2 min (30s × 4) while in an ice-saline bath for cooling. After the remaining cell material was precipitated by centrifugation, lipids were removed from the wet pellet (about 5–6 mg of dry weight) by extraction with a chloroform-methanol (1:2, v/v) mixture (2 × 2 ml). The remaining material was dried under reduced pressure and hydrolyzed in 5.7 N HCl in sealed ampules at 110°C for 24 h. The hydrolyzate was evaporated to dryness and the resulting mixture was derivatized.

Quantitative determination of the excreted amino acids

During fermentation the excreted amino acids were separated by thin-layer chromatography (TLC) on Silufol (Kavalier) plates with mobile phases (A): iso-propanol-25% NH_3 (8:2) and (B): n-butanol-acetic acid-water (4:1:1) using pure commercial amino acids as standard and (C): chloroform-methanol-aceton (14:2:1) for dansyl (Dns-) derivatives. The amount of excreted amino acid was determined for 10 μl aliquots of liquid culture media by TLC in system (A). The spots were detected by 1% ninhydrine solution in ethanol, eluted by 0.5% $CdCl_2$ solution in 50% ethanol (2 ml). The absorbance of the eluates was measured at λ 540 nm and the concentration of the amino acid was determined using calibration curve. For leucine content was further confirmed by quantitative TLC of dansylated sample, chromatographically isolated from the lyophilized culture medium after derivatization using system (C) (the yield of dansylation procedure was taken as 96% and ε 4500 at λ 336 nm).

Derivatization

Benzyloxycarbonyl (Z-) amino acid derivatives were obtained according to standard procedure (Greenstein and Winitz, 1961a), followed esterification by treatment with freshly prepared diazomethane in ether solution (Fieser and Fieser, 1971). Before measuring mass spectra Z-leucine derivatives were purified by column chromatography on silica gel μ 40/100 eluted by chloroform-methanol mixture with 0–10% (v/v) methanol gradient. The samples of lyophilized supernatants and total protein hydrolyzates, as well as standard amino acids, were dansylated in 1M $NaHCO_3$-aceton (1:1) mixture at pH 11, using tenfold excess of dansyl chloride, and treated as described in (Devenyi and Gergely, 1976). To avoid possible by-product formation due to partial methylation of nitrogen of dansylated amino group by diazomethane the esterification of dansyl derivatives was performed with methanol in presence of $SOCl_2$ (Greenstein and Winitz, 1961b).

Equipment

To control the dynamics of cell growth the biophotometer Bonet-Maury-Jouan Biolog-2 (France) was used. Absorbance was measured with a spectrophotometer Beckman DU-6

(USA). Electron impact mass spectrometry was performed on Hitachi MB 80 spectrometer (Japan) at ionizing energy 70 eV and an ion source temperature of 200°C. Each measurement was repeated three times.

Results and discussion

For eight experimental conditions (a–h), shown in the Table 1, biomasses with varying degrees of isotope enrichment were hydrolyzed following lipid removal (see Materials and methods) to control amino acid mixture for retained unexchangeable deuterium atoms or ^{13}C. The hydrogen (deuterium) atoms on amino and carboxylic groups as well as ones on the aromatic rings of amino acids were considered as easily exchangeable. For ^{2}H-labeling various compositions of $C^{2}H_3O^{2}H/^{2}H_2O$ were added to the growth media as hydrogen (deuterium) atoms could be assimilated from water.

Table 1. Isotope components of culture media and some characteristics of bacterial growth

	Media components					Time of generation (min)	Lag-phase (h)	Yield of biomass (%)
	H_2O (%)	$^{2}H_2O$ (%)	CH_3OH (%)	$C^{2}H_3O^{2}H$ (%)				
(a)	100	0	1.0	0		61	0	100
(b)	100	0	0.5	0.5		53	0.2	63
(c)	100	0	0	1.0		60	0.8	51
(d)	50	50	1.0	0		73	2.4	52
(e)	50	50	0.5	0.5		66	5.7	50
(f)	25	75	1.0	0		73	6.7	52
(g)	50	50	0	1.0		74	5.6	47
(h)	100	0	0	1.0	$^{13}CH_3OH$	60	0.1	72

Derivatization to dansyl amino acid methyl esters was used successfully for mass spectrometric analysis of the single amino acids as well as for the lyophilized growth media and the amino acid mixture derived from the total protein hydrolyzates. In addition Z-derivatization was used. This was done for two reasons: firstly, fragmentation of Z-amino acid methyl esters allows not only the confirmation of isotope enrichment, but also the determination of isotope position; secondly, Z- derivatives are convenient for performing an amino acid separation by reverse-phase high performance liquid chromatography. Isotopically labeled Z-amino acids, themselves or after removal of the protective group, are of commercial interest. And finally, Z-derivatization simplified the procedure of isolation of excreted amino acids from the medium significantly decreasing their losses.

According to the comparative mass spectrometric data Z- and Dns- derivatives of leucine derived from conditions (a) were identical to the corresponding derivatives of pure commercial leucine.

The high intensity of peaks corresponding to molecular ions of dansyl amino acid methyl esters (Reshetova et al., 1987) made it possible to obtain interpretable mass spectra of excreted amino acids in the presence of other meta-

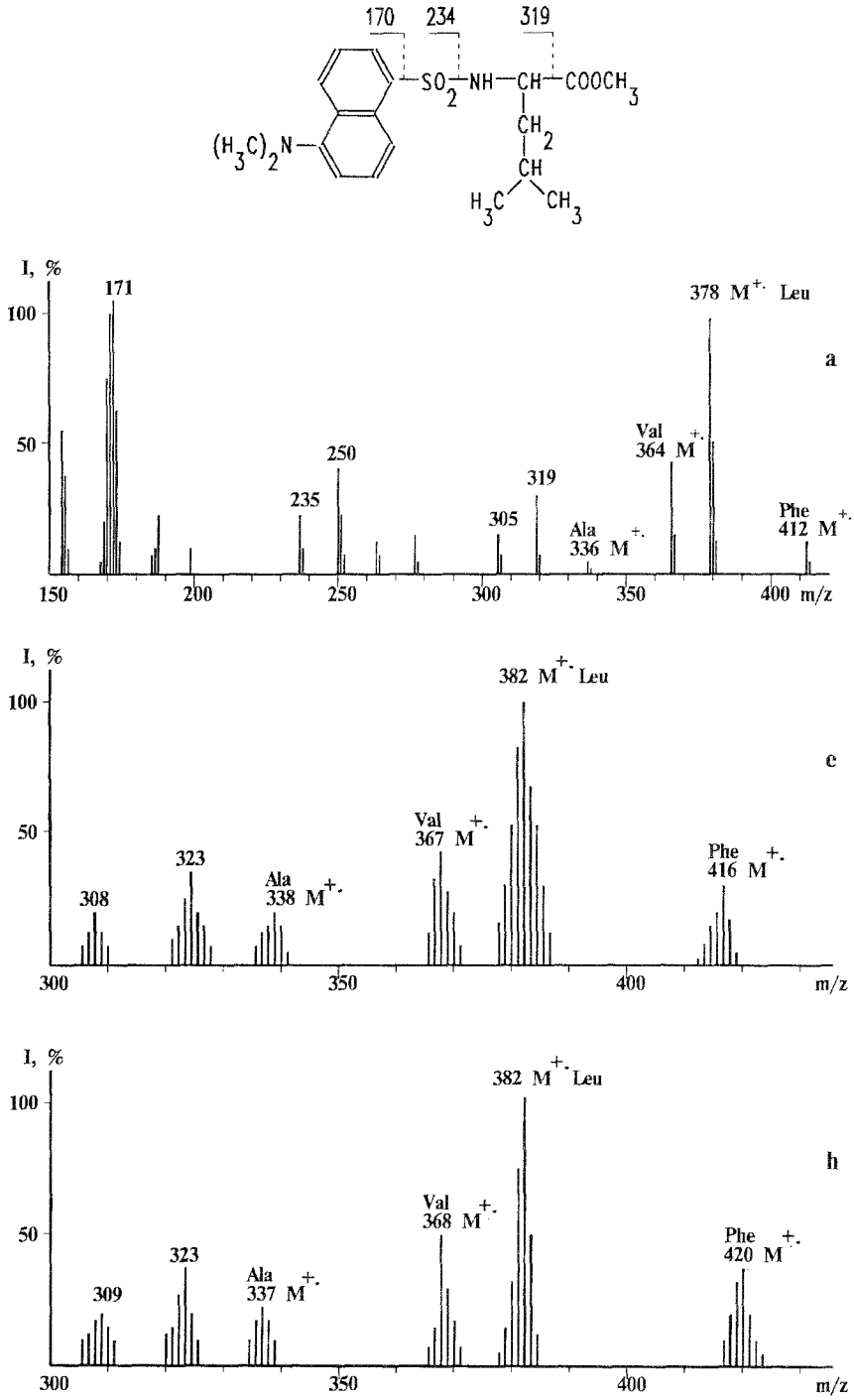
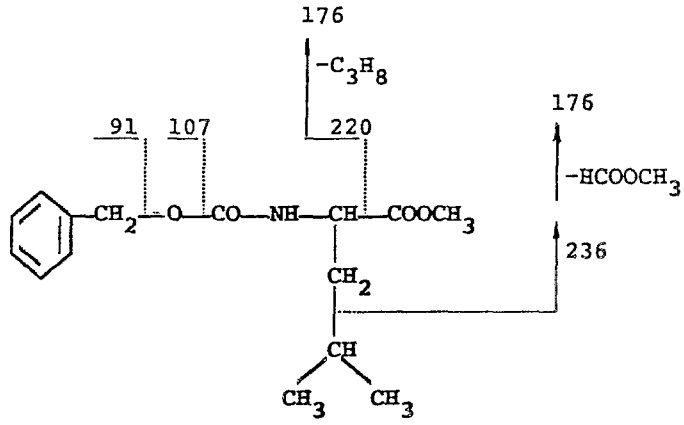


Fig. 1. Mass spectra (70 ev) of dansyl amino acid methyl esters derived from culture media in experiments (**a**), (**e**) and (**h**) respectively

bolites in the growth media. Thus valine (about 3 μM per ml according to the quantitative analysis) as well as trace amounts of alanine and phenylalanine (not detectable by thin layer chromatography) were detected in addition to the main excreted amino acid. Mass spectra of Dns-Leu-OCH_3 derived from non-labeled media as a control is shown in the Fig. 1a. As an example the data on its deuterated analog, derived from growth conditions (e) are shown on the same figure (below). Comparative analysis indicated that biosynthetic isotopic enrichment in those conditions resulted mainly in four deuterium atoms (M^+ at m/z 382 instead of 378 for non-labeled leucine) detected in the leucine molecule after the complete sample preparation procedure. For the alanine molecule mainly two deuteriums corresponded to the highest peak of molecular ion group (M^+ at m/z 338 instead of 336) in the mass spectra and three deuteriums could be detected for valine (M^+ at m/z 367 instead of 364 plus peak at m/z 308 instead of 305 for amino fragment). But nothing can be said about the preferable positions of isotopic enrichment.

The proposed sites of deuterium localization in the leucine molecule could be evaluated from the mass spectrum of Z-Leu-OCH_3 due to fragmentation:



According to the percentage of the peak intensities listed in the Table 2, for the leucine sample from experiment (e) the presence of four unexchangeable deuteriums was confirmed both by the molecular ion (most abundant peak of M^+ at m/z 283 instead of 279) and peaks at m/z 180 (instead of 176) and m/z 224 (instead of 220). The presence of the base peak at m/z 239 (e) instead of 236 (a) proved that the remaining three deuteriums are located generally at the α and β positions. As for methyl groups in the leucine molecule the mass spectra of Z-Leu-OH seem to be more informative (see Fig. 2e) because the peaks at m/z 254 and 209, caused by the cleavage of methyl group, were detected, although in the control spectra (Fig. 1a) the corresponding peaks could be hardly noticed. Altogether these data show the deuteration sites to be mainly α, β and γ positions, but not methyl groups in the leucine molecule. Thus, the use of Dns-derivatives allow the determination of the isotope content due to distribution of peak intensities in the molecular ion group. Z-derivatives can also be informative for elucidation of the positions of isotope enrichment due to typical fragmentation.

Table 2. Electron impact partial mass spectra of Z-Leu-OCH_3 derived from experimental conditions (a) and (e)

Ion designition +	Experimental conditions (a)		(e)	
	m/z	I, %	m/z	I, %
Z-NH-C-CH_2	176	100	176	37.0
	177	20	177	26.0
			178	51.0
			179	87.5
			180	100.0
			181	93.5
			182	60.0
			183	36.0
			184	15.0
$[M-(COOCH_3)]^+$	220	65.5	220	23.0
	221	12.0	221	19.0
			222	26.0
			223	22.0
			224	51.0
			225	43.0
			226	32.0
			227	14.0
$[M-C_3H_7]^+$	236	5.5		
			238	6.0
			239	12.0
			240	6.0
$M^{+\cdot}$	279	11.0	279	5.0
	280	5.0	280	4.0
			281	8.0
			282	11.0
			283	12.5
			284	11.0
			285	6.0

Application of MS monitoring to amino acid mixtures of protein hydrolyzate became the next logical step. Up to 13 amino acid residues could be clearly distinguished in full-scan mass spectrum of non-labeled protein hydrolyzate derivatized into dansyl amino acid methyl esters mixture without any purification procedure (Fig. 3a). In order to identify the peaks and to detect isotopic enrichment of the amino acid mixture all mass spectra of protein hydrolyzates derived from labeled conditions (b–h) were compared to control spectra and to spectra of corresponding derivatized growth media containing excreted amino acids. As an illustration mass spectrum of derivatized protein hydrolyzate (without any special purification) from experiment (e) is shown in the Fig. 3.

The trends of isotope enrichment of amino acid residues of total protein could be represented by the main ions M^+ as the mass-to-charge ratio of

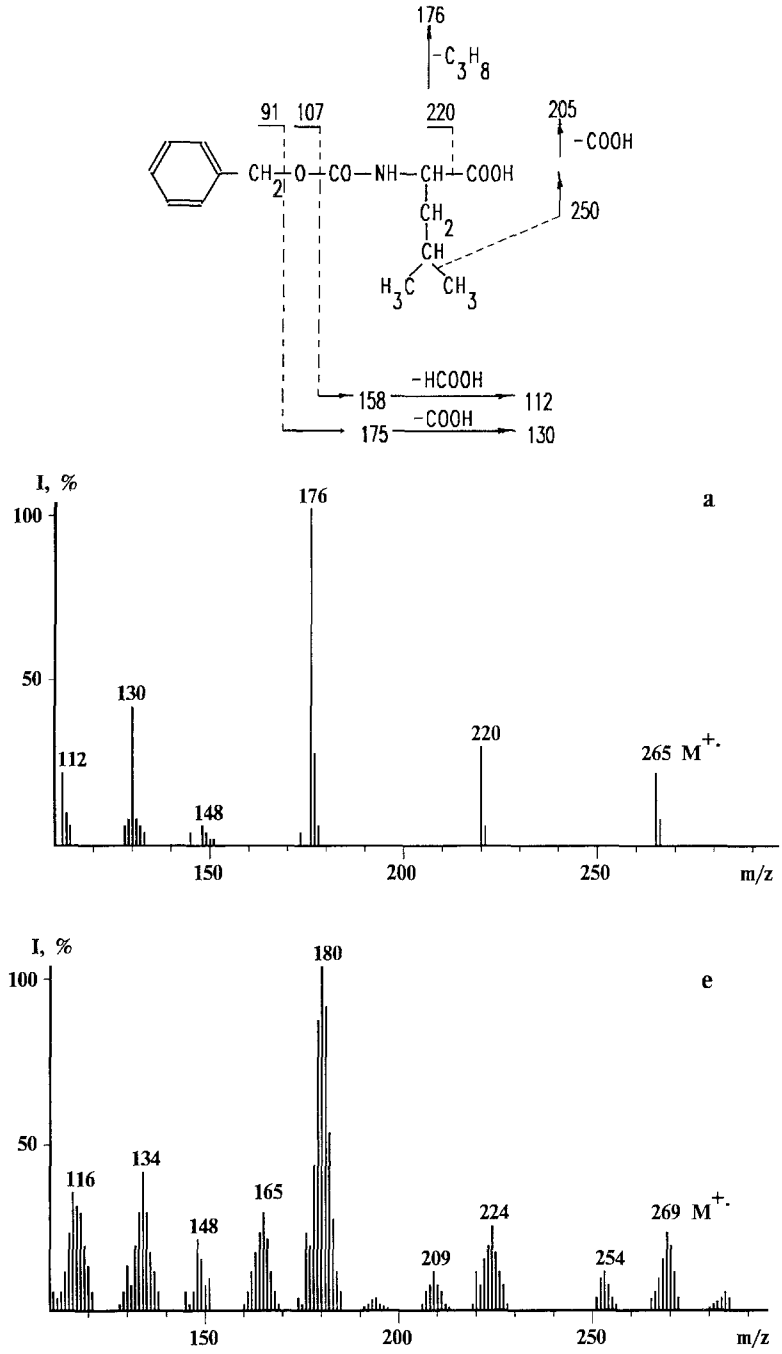


Fig. 2. Mass spectra of benzyloxycarbonyl leucine derived from culture media in experiments (**a**) and (**e**) respectively

deuterated and non-deuterated species. For example for protein hydrolyzate in experimental conditions (e) ^{2}H-enrichment can be described as:

Compound	m/z (a)	$(M^{+\cdot})$ (e)	Compound	m/z (a)	$(M^{+\cdot})$ (e)
Dns-Gly-OMe	322	322	Dns-Asp(OMe)-OMe	394	395
Dns-Ala-OMe	336	338	Dns-Glu(OMe)-OMe	408	410
Dns-Ser-OMe	352	353	Dns-Phe-OMe	412	416
Dns-Val-OMe	364	367	Dns-Lys(Dns)-OMe	626	629
Dns-Leu/Ile-OMe	378	382	Dns-Tyr(Dns)-OMe	661	663

The peaks of the molecular ions of dansyl proline methyl ester (most abundant peak at m/z 362) and dansyl threonine methyl ester (m/z 366) are overlapped by the cluster of derivatized valine (m/z 364).

Base peak M^+ (at m/z 338) of alanine indicated that mainly two deuteriums were introduced into the molecule (338 instead of 336), the adjacent peaks at m/z 337 and 336 characterize the impurities of monolabeled and nonlabeled molecules.

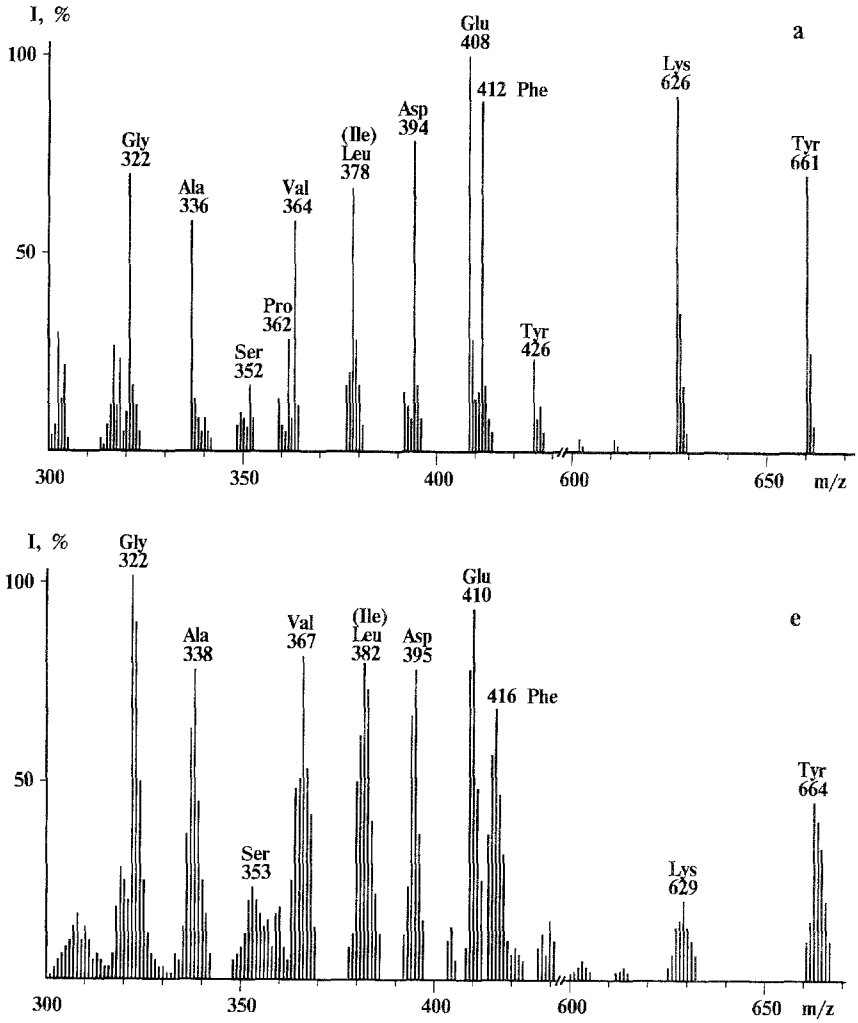


Fig. 3. Mass spectra of dansyl amino acid methyl esters mixtures of protein hydrolyzates from experiments (**a**), (**e**) respectively

Possible reverse ^{2}H-H exchange during acid hydrolysis can result in the loss of deuteriums by aromatic rings of amino acids. Thus for labeling in conditions (e) mainly two deuteriums retained in the tyrosine molecule were detected by mass spectrometric analysis after hydrolysis in HCl. The peaks of molecular ions both of mono- and di-dansyl derivatives of tyrosine methyl ester confirmed the presence of these two deuteriums which can be attributed to α and β positions, while in the case of phenylalanine mainly four deuteriums were detected, which could mean that at least one of them should be attributed to the aromatic ring. As expected, no traces of tryptophan were found in the hydrolyzates as it should be determined by alkaline hydrolysis (Karnaukhova et al., 1989; Raap et al., 1990).

The correlations between biosynthetic isotope incorporation into the alanine, valine and leucine or alanine, serine and aspartic acid were noticed. At the same time the different isotope enrichment detected for tyrosine and phenylalanine needs to be further explained.

The data on leucine and valine from protein hydrolyzate are in excellent agreement with the data on the same amino acids excreted into culture medium during methylotroph growth. Thus, the analysis of excreted amino acids can be used to evaluate biosynthetic isotope enrichment of cell protein.

The utilizing of obligate methylotrophs for the labeling with ^{13}C-methanol is the most attractive and reasonable use of them when other carbon sources are excluded. The mass spectra of derivatized ^{13}C-labeled amino acids excreted into the culture media is presented in the Fig. 1 (h). According to the relative peak intensities mainly four ^{13}C atoms (among six) characterize biosynthetic enrichment of excreted leucine (peak at m/z 382 instead of 378). Besides the main product, the species with five and six isotope atoms, as well as less enriched, are present. Similarly, mainly four ^{13}C atoms (among five) could be detected for valine and eight atoms (among nine) for phenylalanine.

Labelling experiments with obligate methylotrophs based on ^{13}C-methanol bioconversion have other important feature, namely, ^{13}C-labeled compounds, compared with deuterated ones, are less subjected to exchange reactions and the level of ^{13}C-enrichment can be easily detected by MS. Unfortunately the price of ^{13}C-labeled methanol should be taken into account for the large scale production of the labeled compounds.

The data obtained suggest that using the obligate methylotroph, *Methylobacillus flagellatum*, for preparation of stable isotope labeled amino acids is feasible. Up to 12 μmol of excreted ^{2}H- or ^{13}C-labeled leucine (isoleucine, phenylalanine, threonine or tryptophan for various mutants) per ml of culture medium can be isolated. Other labeled amino acids can be separated from total protein hydrolyzates. As it was shown with the aid of MS analysis the levels of isotope incorporation into the excreted amino acids and the analogous amino acid residues in the total protein were the same and the excreted amino acids can be used as an indicator of isotopic enrichment of total protein in express analysis.

Comparative data testify to high efficiency of dansyl derivatization for electron impact mass spectrometric analysis of complex amino acid mixtures both of culture media and cell protein hydrolyzates.

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