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Application of ^{13}C-[2] - and ^{13}C-[1,2] acetate in metabolic labelling studies of yeast and insect cells

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Abstract

The advantage of using ^{13}C-labelled glucose in metabolic studies is that it is an important carbon and energy source for almost all biotechnologically and medically important organisms. On the other hand, the disadvantage is its relatively high cost in the labelling experiments. Looking for cheaper alternatives we found that ^{13}C-[2] acetate or ^{13}C-[1,2] acetate is a prospective compound for such experiments. Acetate is well incorporated by many organisms, including mammalian and insect cell cultures as preferred source of acetyl-CoA. Our experimental results using ^{13}C NMR demonstrated that acetate was efficiently incorporated into glutamate and alanine secreted by the insect cell culture. Using D-stat culture of *Saccharomyces uvarum* on glucose/^{13}C-acetate mineral media we demonstrated that the labelling patterns of proteinogenic amino acids can be well predicted on the basis of specific substrate consumption rates using the modified scheme of yeast metabolism and stoichiometric modelling. According to this scheme aspartate and alanine in *S. uvarum* under the experimental conditions used is synthesised in the mitochondria. Synthesis of alanine in the mitochondria was also demonstrated for *Spodoptera frugiperda*. For both organisms malic enzyme was also operative. For *S. uvarum* it was shown that the activity of malic enzyme is sufficient for supporting the mitochondrial biosynthetic reactions with NADPH.

Abbreviations: BDF – biosynthetically directed fractional labelling; FCS – foetal calf serum; IEAM – yeast extract free medium; MEM – minimal essential medium; MFA – metabolic flux analysis; oxa_c – cytosolic oxaloacetate; oxa_m – mitochondrial oxaloacetate; PP pathway – pentose phosphate pathway; Pyr_c – cytosolic pyruvate; Pyr_m – mitochondrial pyruvate; TCA – tricarboxylic acid

Introduction

Integration of the information obtained from the databases of the different fields of modern biotechnology together with the data of labelling experiments can be used for composing mathematical models describing either the entire metabolism or certain pathways of a given organism under defined environmental conditions. Different strategies of labelling studies expressing the distinct feature of metabolic network analysis for different organisms have been proposed by several

authors (Chance et al. 1983; Christensen and Nielsen 1999; Dauner and Sauer 2001; Christensen et al. 2002). It is widely accepted that the mathematical description of the biochemical processes in the cell requires the mass balancing of the participating metabolic reactions through the key intermediates. A powerful technique for metabolite balancing is metabolic flux analysis (MFA) which uses the set of the measured fluxes such as substrate consumption rate(s), product excretion rate(s) and the exact biomass composition to establish a determined system that can be solved by simple linear algebra (Vilu et al. 1990; Vallino and Stephanopoulos 1993). Most widely used alternatives are linear optimization if the system cannot be determined, and labelling (isotope) balancing (Varma and Palsson 1994; Wiechert 2001).

The metabolite balancing combined with labelling experiments and isotope balancing enables gathering of additional information on overall metabolic topology as the response to the environmental conditions or genetic manipulations of the organism. Labelling experiments have been shown to be an efficient tool for tracing the active metabolic reactions in the cells (Barton et al. 1982; Paalme et al. 1982; Walker et al. 1982). Moreover, it enables the determination of fluxes in different compartments of the cells, which is especially important for the characterization of the active metabolic pathways in eukaryotic organisms. In the labelling experiments the substrate with defined labelling state (e.g. 1–^{13}C-glucose or mixture of uniformly labelled ^{13}C-glucose and unlabelled glucose) is fed to the microbial or cell culture and the following distribution of the labelled carbon atoms between the different positions of the metabolites is identified either by NMR or GC-MS, thus visualizing the exact topology of the active metabolic pathways. To identify the labelling state of the intermediates that are usually not stable and are present in relatively small concentrations being difficult to determine, the ^{13}C NMR-spectra of corresponding proteinogenic amino acids and components of nucleic acids and polysaccharides have been used (Paalme et al. 1982; Szyperski et al. 1996; Maaheimo et al. 2001). In order to simplify the modelling and as the observed microbial culture or cell line system should be kept in a well defined stationary physiological state during the measurement procedures, steady state conditions would be preferred in labelling experiments (Wiechert and de Graaf 1996).

In most of the studies to date the labelling pattern of the carbon atoms of the intermediates, which have been obtained from the labelling experiments using labelled glucose have been used as inputs to the model for calculating the metabolic flux values (Christensen and Nielsen 1999; Maaheimo et al. 2001; Christensen et al. 2002; Cannizzaro et al. 2004). Despite the higher cost of labelled glucose, the advantage of its application in labelling experiments is that it is the most important carbon and energy source for most of the biotechnologically important (micro)organisms. As an alternative, labelled acetate along with unlabelled glucose has been used as an effective and relatively low cost substrate (Dickinson et al. 1983; Tran-Dinh et al. 1996; dos Santos et al. 2003).

In the present paper we introduce a unique modelling strategy to elucidate the time-spatial structure of *S. uvarum* and *Spodoptera frugiperda* (Sf9) metabolism using *D-stat* cultivation technique (Kasemets et al. 2003) enabling more convenient sampling than chemostat for labelling experiments with ^{13}C-labelled acetate.

Materials and methods

Mathematical framework of the model of label movement

The metabolite balances as a system of stoichiometric equations were composed on the basis of the metabolic network, based on the results of the previous labelling experiments of other authors (Maaheimo et al. 2001; dos Santos et al. 2003; dos Santos et al. 2004), genome databases such as Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.com/) and Saccharomyces Genome Database (http://www.yeastgenome.org/), and taking into account the labelling patterns of amino acids obtained in this work. Fluxes through the intermediates (Figure 1) were calculated for the given cultivation conditions, supposing that in *D-stat* culture the intracellular concentration of metabolites remains constant in time:

$$\text{fluxes into the intermediate} - \text{fluxes out of the intermediate} = \frac{dS_i}{dt} \qquad \left(\frac{dS_i}{dt}\right) = 0 \quad (1)$$

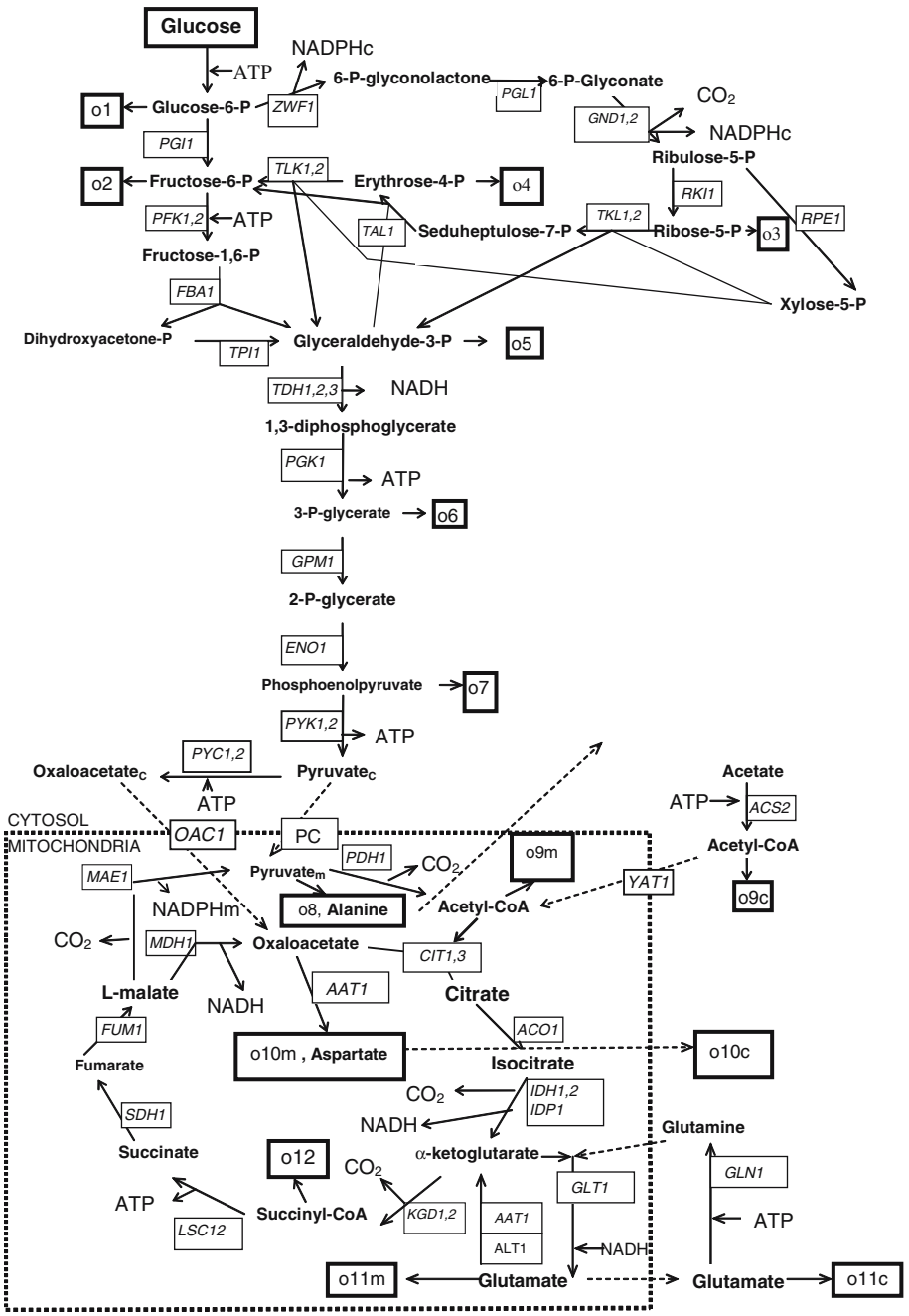

Figure 1. Metabolic network of active pathways in *S. uvarum* grown on glucose and acetate. Fluxes into the intermediates are given *in italics*, expressed as the gene abbreviation catalyzing the reaction; fluxes leading to the biomass components (Cortassa, 1995) are expressed as o_i: $\boldsymbol{o}_1$, polysaccharides; $\boldsymbol{o}_2$, peptidoglucans (not determined); $\boldsymbol{o}_3$, His, dAMP, dGMP, dUMP and dCMP; $\boldsymbol{o}_4$, Phe, Trp and Tyr; $\boldsymbol{o}_5$, glycerolphosphate; $\boldsymbol{o}_6$, Cys, Gly, dAMP and dGMP; $\boldsymbol{o}_7$, Phe, Trp and Tyr; $\boldsymbol{o}_8$, Ala, Ile, Leu and Val; $\boldsymbol{o}_{9c}$, Lys, Ser, Met, lauric acid, palmitoleic acid and oleic acid; $\boldsymbol{o}_{9m}$, Leu; $\boldsymbol{o}_{10m}$, Asp and Ile; $\boldsymbol{o}_{10c}$, Met, Thr, Asn, dUMP and d CMP; $\boldsymbol{o}_{11m}$, Glu and Arg; $\boldsymbol{o}_{11c}$, Gln, Pro and Lys; $\boldsymbol{o}_{12}$, heme compounds (not determined); fluxes between the compartments are marked with dashed arrows.

The analytical solution of the system written on the basis of Figure 1 for $MAE1 = o_{NADPm}$ is given in Appendix A. Values for the consumption of key intermediates (o_i) for cellular biosynthesis were calculated based on the macromolecular composition of biomass (protein, lipids, nucleic acids and bi- and polysaccharides) according to the monomer composition of the polymers as described in Cortassa et al. (1995). The ^{13}C enrichments of the carbons of key intermediates were calculated using the corresponding flux values obtained from the stoichiometric equation system and labelling patterns of acetate and glucose as well as the scheme describing the label movement (Figure 3 for *S. frugiperda*, not shown for yeast):

$$p_i = \frac{{p_A}^i * \mathrm{flux_A} + {p_B}^i * \mathrm{flux_B}}{\mathrm{flux_A} + \mathrm{flux_B}} \quad (2)$$

$$p_{ij} = \frac{{p_A}^{ij} * \mathrm{flux_A} + {p_B}^{ij} * \mathrm{flux_B}}{\mathrm{flux_A} + \mathrm{flux_B}} \quad (3)$$

where p_i is the probability of ^{13}C labelling of position i in the compound under study; p_{ij} is the probability of labelling of both carbons i and j; ${p_A}^i$ and ${p_B}^i$ (or ${p_A}^{ij}$ and ${p_B}^{ij}$) are the probabilities of enrichment of corresponding carbons in precursor molecules A and B and $\mathrm{flux_A}$, $\mathrm{flux_B}$ are the corresponding fluxes from the precursor molecules.

Organisms and cultivation conditions of labelling experiments

Commercially produced brewer's yeast strain *S. uvarum* W34 was kindly provided by Dr R. Degré from Lallemand, Inc. Culture was maintained on YEDC agar (Lab M, UK) at +4 °C prior the use. Pure yeast culture from the agar plates was inoculated into a 50 ml flask with sterilized inoculation medium composed of (g l^{-1}): malt extract (Lab M, UK), 10; NaCl, 5; and glucose (Difco), 2. Preculture was grown in the flask on a shaker at 30 °C for 24 h following inoculation into the culture vessel. For labelling experiments the *D-stat* (Kasemets et al. 2003) with changing culture volume at dilution rates of 0.04 h^{-1} and 0.08 h^{-1} was used in order to simplify the biomass collection. A homemade culture vessel with working volume 150–250 ml as described in Drews et al. (2003) was used. The cultivation temperature was 30 °C, pH was kept at 4.5 by titration with 1 N HCl and pO_2 was kept above 60% of saturation by spraying the air through a 0.2 μm microfilter (Whatman).

About 10 ml of culture grown in the flask was inoculated into the culture vessel filled with *in situ* sterilized 140 ml of defined dilution media (Paalme et al. 1997) containing unlabelled glucose (3 g l^{-1}) and Na acetate (1 g l^{-1}) as the carbon sources. After the complete consumption of glucose the regular *chemostat* cultivation was started. In order to obtain the steady state culture conditions 5 culture volumes of the dilution media containing unlabelled Na-acetate and glucose were passed through the culture vessel. Then the dilution media was changed to the one containing either [1,2]–^{13}C Na-acetate (1.06 g l^{-1}) and unlabelled glucose (2.6 g l^{-1}) or [2]–^{13}C Na-acetate (0.76 g l^{-1}) and unlabelled glucose (2.8 g l^{-1}), and the constant outflow from the fermenter was stopped. The pumping rate of the dilution media was increased according to the increasing culture volume to maintain the constant dilution rate (Drews et al. 2003). When the culture volume reached 250 ml, 100 ml of culture liquid was pumped out within 1 min and the cultivation was continued at the volume of 150 ml again. A sufficient amount of biomass for determination of the labelling pattern and ^{13}C enrichments in the proteinogenic amino acids was obtained after repeating the procedure 12 times. When the labelled biomass had been collected, a similar procedure was carried out with the unlabelled biomass (grown on glucose and unlabelled Na-acetate) for quantification of the cell biomass components.

Spodoptera frugiperda Sf9 cells were originally obtained from KaroBio AB (Sweden). Cells were maintained at 27 °C in 25 cm^2 flasks. For labelling experiment the cells were adapted to in-house made medium for 1.5 months. The yeast extract free medium containing 5x essential amino acid solution of minimal essential medium (MEM) (Sigma), 10% FCS (*foetal calf serum*, Gibco), 0.2% Pluronic-F-68 (Sigma), glucose, 5 g l^{-1}; glutamine, 1 g l^{-1}; serine, 0.55 g l^{-1}; lysine, 0.65 g l^{-1}; proline, 0.35 g l^{-1} and glycine, 0.2 g l^{-1}. The penicillin/streptomycin stock (Hybrimax) was used for antibiotics. The composition of the trace elements and vitamins was the same as decribed in Weiss et al. (1981). For the labelling experiment 0.5 g l^{-1}

of ^{13}C-[2] Na-acetate was added into the medium and the short term (48 h) batch experiment was conducted in a 1 l spinner flask (Bellco) with culture volume of 400 ml.

Analytical methods

Biomass concentration in the labelling experiments with *S. uvarum* was determined by measuring the optical density at 540 nm as described in Adamberg et al. (2003) every time 100 ml of biomass fraction was withdrawn from the culture vessel. Cell numbers of *Spodoptera frugiperda* Sf9 were measured by hemocytometer and viability by Trypan Blue exclusion. The glucose and acetate concentrations in the culture and feeding media were determined using high pressure liquid chromatography (HPLC). Isocratic elution (0.6 ml min^{-1}) of Aminex HPX-87A (Biorad) column (300 × 7.8 mm and particle size of 9 μm) with 0.09 N H_2SO_4 was used. UV_{206} and RI detectors were applied in parallel.

The yeast biomass fractions withdrawn from the culture vessel were collected directly into the 2× 50 ml centrifuge tubes on ice bath and separated by centrifugation at 20,000 *g* at 4 °C. Biomass from each fraction was resuspended and washed twice with ice cold distilled water and concentrated into eppendorfs (in duplicates from each fraction). One of the duplicates from the fractions with nonlabelled biomass was used to determine the biomass dry weight for exact quantification of cellular components. Trehalose was determined by the modified method described in Ferreira et al. (1997) and Kylie et al. (1987) using the extraction with hot ethanol and determination by HPLC. Glycogen was extracted from the cell debris left after the trehalose extraction as described in Gerhardt et al. (1994) and hydrolyzed in 1 N H_2SO_4 at 100 °C for 1 h. The liberated glucose was determined by HPLC. Cell membrane polysaccharides were hydrolyzed as described in Dallies et al. (1998) and liberated monomers (glucose, mannose and glucosamine) were determined by HPLC. Biomass lipids were extracted with hexane–isopropanol solution (3:2) as described in Radin (1981), the extract was dried in gaseous nitrogen flow and the dry weight of total lipids was determined. *Amino acids and protein content*: cells (^{13}C-labelled and unlabelled) were hydrolyzed in 6 ml 6 N HCl for 24 h at 110 °C in sealed glass tubes under vacuum. The biomass debris was washed twice with distilled water and hydrolysate was analyzed for amino acids with the amino acid analyzer (Biochrom, UK) using lithium citrate buffers and post column derivatization with ninhydrin. Total protein content was determined as the sum of the proteinogenic amino acids from the biomass. *Nucleic acids and deoxyribonucleotide composition*: RNA and DNA were extracted from the cells using 0.5 N $HClO_4$ at 70 °C for 15 min and determined as described in Gerhardt et al. (1994). The composition of deoxyribonucleotides was calculated based on the data of Cortassa et al. (1995).

NMR measurements and interpretation of ^{13} C-labelled amino acid spectra. Determination of labelling pattern from ^{13}C NMR spectra of biomass components

^{13}C-NMR spectra were obtained on a Bruker AMX500 pulsed Fourier transform spectrometer operating at 125.7 MHz under conditions of complete proton composite pulse (waltz16) decoupling and with internal deuterium field lock. Free induction decays were accumulated as 64 K data points with Fourier transformations in 256 K. The bandwith of 30 kHZ and about 30 degree 2 (μs) observation pulses with a relaxation delay of 1 s were used. 2D Fourier transform spectra were obtained with the standard Bruker pulse programs. Both 1D and 2D spectra were processed using "Mestre-C" software (http://www.mestreC.com/). Carbon atom multiplets of the different amino acids were determined based on their chemical shift values described in Tran-Dinh et al. (1973) and Wütrich (1976). The NMR spectra of the hydrolyzed labelled biomass fraction were used to calculate the experimental absolute and conditional enrichments of proteinogenic amino acids. As in proton-decoupled ^{13}C NMR spectra each carbon peak intensity is proportional to the number of labelled carbon atoms in the sample, the total integral of the multiplet of the given carbon position of the amino acid was divided by the relative concentration of the amino acid to calculate the absolute ^{13}C enrichments. The enrichment of C_α of Lys was taken equal to the enrichment of C_α of acetate and used as the internal standard. In parallel, the mixture of

compounds under study with natural enrichment was prepared and analyzed by ^{13}C NMR. The absolute enrichment (E_j) of the carbon j (C_j) of the amino acid under study was calculated using the formula:

$$E_j = \frac{E_{st} * M_j * C_{st}}{M_{st} * C} * \frac{M'_{st} * C'}{M'_j * C'_{st}} \quad (4)$$

where E_{st} is the enrichment of C_α of Lys; M_j is the total intensity (integral) of the multiplet peaks of C_j in the sample; C_{st} is the concentration of the Lys in the sample; M_{st} is the total intensity (integral) of Lys C_α multiplet peaks in the sample; C is the concentration of the amino acid under study in the sample; M'_{st} is the total intensity (integral) of Lys C_α multiplet peaks in the calibration sample; C' is the concentration of the amino acid in the calibration sample; M'_j is the total intensity (integral) of the multiplet peaks of C_j in the calibration sample and C'_{st} is the concentration of Lys in the calibration sample.

Calculation of the conditional enrichments (Paalme et al. 1982) in two-carbon system (E_i^j), showing the enrichment of C_i of the amino acid under study in the molecules labelled also in C_j was carried out as followed:

$$E_i^j = \frac{D_{ji} + Q_{jik}}{D_{ji} + D_{jk} + Q_{jik} + S_j} \quad (5)$$

where D_{ji} is the doublet intensity (integral) of C_j of the amino acid under study, arising from coupling with the adjacent C_i; D_{jk} is the doublet intensity (integral) of C_j of the amino acid under study, arising from coupling with the adjacent C_k; Q_{jik} is the quartet intensity (integral) of C_j of the amino acid under study, arising from coupling with the adjacent C_k and S_j is the intensity (integral) of the singlet of C_j of the same amino acid.

Results and discussion

The steady state culture of *S. uvarum* was obtained on mineral glucose media with ^{13}C-[2] or ^{13}C-[1,2] Na acetate. The ethanol-soluble fraction of biomass containing mainly trehalose and glutamate, and insoluble fraction containing amino acids and nucleotides was separated from the cells. The insoluble fraction was hydrolyzed to determine the distribution of the label in the proteinogenic amino acids. The label from acetate was observed in Glu, Ala, Thr, Asp, Pro, Arg, Lys, Leu and Ileu. Incorporation of the label into other amino acids could not be determined. The labelling pattern and ^{13}C enrichments of amino acids were compared with those expected according to the scheme of the label movement for *S. cerevisiae* suggested by Maaheimo et al. (2001) and dos Santos et al. (2003). The labelling patterns obtained in our experiments with *S. uvarum* were generally comparable with this scheme, except for the localization of the synthesis of Asp. Based on the absolute and conditional enrichments calculated from the ^{13}C NMR spectra the modified scheme was postulated (Figure 1): (i) Asp is synthesized from the mitochondrial pool of oxaloacetate in mitochondrial aspartate transaminase reaction (*AAT1*) and transported out from the mitochondria for synthesis of Asn, Thr and Met, as well as pyrimidines; (ii) Ala is synthesized in the mitochondria by alanine aminotransferase (*ALT1*); (iii) mitochondrial NADPH is derived exclusively in the reaction of malic enzyme (*MAE1*) and (iv) in the given conditions Glu required for transamination of mitochondrial oxaloacetate and pyruvate is mainly synthesized via NADH-dependent glutamate synthase (*GLT1*).

According to the modified scheme we composed the stoichometric model of label movement in *S. uvarum* and calculated the labelling pattern of the key intermediates on the basis of substrate consumption rates, biomass yield and macromolecular composition of the biomass (Table 1). The monomer composition of the macromolecular fraction and the specific NADPH requirement for their synthesis were calculated as described in Cortassa et al. (1995). The analytical expressions of the labelling patterns are given in the Appendix B.

Comparison of the absolute and conditional enrichments of proteinogenic amino acids and their precursors calculated from the model and those of calculated from ^{13}C NMR spectra (Tables 2 and 3) showed very good agreement. A similar fit for the enrichments of Asp with the scheme proposed by Maaheimo et al. (2001) can be obtained only in case the mitochondrial barrier for oxaloacetate does not operate or the oxaloacetate flux from the mitochondria into the cytosol ($OAC1_{-1}$) has much higher absolute value than the resulting flux $OAC1 = OAC1_{+1} + OAC1_{-1}$. This

Table 1. The basic input values used in the stoichiometric model for the experiments using unlabelled glucose and ^{13}C-[1,2] or ^{13}C-[2] Na-acetate in the dilution media.

	^{13}C-[1,2] Na-acetate (99%) + glucose	^{13}C-[2] Na-acetate (90%) + glucose
Dilution rate [h^{-1}]	0.04	0.08
Acetate consumption rate [mMol g^{-1} dwt]	11	6
Glucose consumption rate [mMol g^{-1} dwt]	12	10
Biomass yield [g dwt g^{-1} substrate]	0.348	0.447
Protein	45	45
Total carbohydrates	30	30
Lipids	11	11
Nucleic acids	5.4	5.4
Ash	8.6	8.6

Table 2. Comparison of theoretically calculated absolute and conditional enrichments of the key intermediates of amino acid metabolism and corresponding carbon atoms calculated from ^{13}C NMR spectra of proteinogenic amino acids in the experiment with ^{13}C-[1,2] Na-acetate[1].

Precursor carbon (model)	Corresponding carbons in proteinogenic amino acids (experimental)					
Ace-CoA$_C$ C_0 = 99%	Lys C_0 = 98%					
Ace-CoA$_C$ C_α = 99%	Lys C_α = 97%					
Ace-CoA$_m$ C_α = 42%	Leu C_α = 42%					
oxa$_m$ C_0 = 29% (1.1%)		Thr C_0 = 28%		Ile C_0 = 30%		
oxa$_m$ C_α = 29% (1.1%)		Thr C_α = 31%		Ile C_α = 33%		
oxa$_m$ C_β = 29% (1.1%)		Thr C_β = 32%		Ile $C_{\gamma 1}$ = 24%		
oxa$_m$ C_γ = 33% (23%)	Asp C_γ = 32%	Thr C_γ = 35%		Ile C_δ = 32%		
Pyr$_m$ C_α = 5%	Ala C_α = 5%	Leu C_β = 5%		Val C_α = 5%	Val C_β = 7%	Ile C_β = 7%
Pyr$_m$ C_β = 5%	Ala C_β = 5%	Leu $C_{\delta 1}$ = 6%	Leu $C_{\delta 2}$ = 6%	Val $C_{\gamma 1}$ = 6%	Val $C_{\gamma 2}$ = 5%	Ile $C_{\gamma 2}$ = 4%
2 kg$_m$ C_0 = 33%	Glu C_0 = 36%					
2 kg$_m$ C_α = 29%	Glu C_α = 28%					Lys C_β = 35%
2 kg$_m$ C_β = 29%		Arg C_β = 33%				Lys C_γ = 29%
2 kg$_m$ C_γ = 42%		Arg C_γ = 37%		Pro C_γ = 35%		Lys C_δ = 42%
2 kg$_m$ C_δ = 42%		Arg C_δ = 42%		Pro C_δ = 37%		Lys C_ε = 43%
Ace-CoA$_C$ C_α^0 = 99%	Lys C_α^0 = 99%					
Ace-CoA$_C$ C_0^α = 99%	Lys C_0^α = 99%					
Ace-CoA$_m$ C_α^0 = 94%	Leu C_α^0 = 93%					
oxa$_m$ C_α^0 = 69% (1.1%)	Asp C_α^0 = 68%	Thr C_α^0 = 68%		Ile C_α^0 = 68%		
oxa$_m$ C_0^α = 69% (1.1%)	Asp C_0^α = 74%					
oxa$_m$ C_β^γ = 61% (1.1%)		Thr C_β^γ = 64%		Ile $C_{\gamma 1}^\delta$ = 64%		
oxa$_m$ C_γ^β = 69% (23%)				Ile $C_\delta^{\gamma 1}$ = 62%		
Pyr$_m$ C_α^0 = 58%	Ala C_α^0 = 54%			Val C_α^0 = 54%		
Pyr$_m$ C_α^β = 29%	Ala C_α^β = 33%	Leu $C_\gamma^{\delta 1}$ = 34%		Val $C_\beta^{\gamma 1}$ = 33%		Ile $C_\beta^{\gamma 2}$ = 31%
2 kg$_m$ C_α^0 = 61%	Glu C_α^0 = 58%	Pro C_α^0 = 61%		Arg C_α^0 = 61%		
2 kg$_m$ C_0^α = 69%	Glu C_0^α = 73%					
2 kg$_m$ C_β^γ = 29%	Glu C_β^γ = 35%	Pro C_β^γ = 26%				
2 kg$_m$ C_γ^δ = 94%	Glu C_γ^δ = 92%	Pro C_γ^δ = 94%		Arg C_γ^δ = 94%		
2 kg$_m$ C_δ^γ = 94%	Glu C_δ^γ = 92%	Pro C_δ^γ = 87%		Arg C_δ^γ = 88%		

[1] Ace-CoA$_C$ and Ace-CoA$_m$, cytosolic and mitochondrial acetyl-CoA; oxa$_m$, mitochondrial oxaloacetate; Pyr$_m$, mitochondrial pyruvate; 2kg$_m$; mitochondrial 2-ketoglutarate.
Numbers in parentheses indicate the fractional enrichments of cytosolic oxaloacetate.

seems to us during respiratory growth, however, very unlikely. In addition, it was supposed in our model that the requirement of NADPH for the mitochondrial biosynthesis is fully covered by the reaction of *MAE1*. Based on our experimental data, the latter can only be possible if NADPH is

Table 3. Comparison of theoretically calculated absolute and conditional enrichments of the key intermediates of amino acid metabolism and corresponding carbon atoms calculated from ^{13}C NMR spectra of proteinogenic amino acids in the experiment with ^{13}C-[2] Na-acetate[1].

Precursor carbon (model)	Corresponding carbons in proteinogenic amino acids (experimental)					
Ace-CoA$_C$ $C_\alpha = 90\%$	Lys $C_\alpha = 90\%$					
Ace-CoA$_m$ $C_0 = 3\%$	Leu $C_0 = 3\%$					
Ace-CoA$_m$ $C_\alpha = 28\%$	Leu $C_\alpha = 28\%$					
oxa$_m$ $C_0 = 6\%$ (1.1%)	Asp $C_0 = 8\%$	Thr $C_0 = 6\%$		Ile $C_0 = 11\%$		
oxa$_m$ $C_\alpha = 15\%$ (1.1%)	Asp $C_\alpha = 20\%$	Thr $C_\alpha = 15\%$		Ile $C_\alpha = 15\%$		
oxa$_m$ $C_\beta = 15\%$ (1.1%)	Asp $C_\beta = 22\%$	Thr $C_\beta = 14\%$		Ile $C_{\gamma 1} = 15\%$		
oxa$_m$ $C_\gamma = 8\%$ (5%)[2]	Asp $C_\gamma = 8\%$	Thr $C_\gamma = 7\%$		Ile $C_\delta = 7\%$		
Pyr$_m$ $C_0 = 2\%$	Ala $C_0 = 3\%$					
Pyr$_m$ $C_\alpha = 4\%$	Ala $C_\alpha = 3\%$	Leu $C_\beta = 2\%$		Val $C_\alpha = 3\%$	Val $C_\beta = 3\%$	Ile $C_\beta = 2\%$
Pyr$_m$ $C_\beta = 4\%$	Ala $C_\beta = 3\%$	Leu $C_{\delta 1} = 4\%$	Leu $C_{\delta 2} = 4\%$	Val $C_{\gamma 1} = 3\%$	Val $C_{\gamma 2} = 4\%$	Ile $C_{\gamma 2} = 4\%$
2 kg$_m$ $C_0 = 8\%$	Glu $C_0 = 8\%$	Arg $C_0 = 11\%$				
2 kg$_m$ $C_\alpha = 15\%$	Glu $C_\alpha = 16\%$	Arg $C_\alpha = 11\%$				Lys $C_\beta = 18\%$
2 kg$_m$ $C_\beta = 15\%$	Glu $C_\beta = 14\%$	Arg $C_\beta = 14\%$				Lys $C_\gamma = 14\%$
2 kg$_m$ $C_\gamma = 28\%$	Glu $C_\gamma = 24\%$	Arg $C_\gamma = 23\%$		Pro $C_\gamma = 25\%$		Lys $C_\delta = 24\%$
2 kg$_m$ $C_\delta = 3\%$	Glu $C_\delta = 2\%$	Arg $C_\delta = 4\%$		Pro $C_\delta = 2\%$		Lys $C_\varepsilon = 3\%$
oxa$_m$ $C_\alpha^0 = 18\%$ (1.1%)	Asp $C_\alpha^0 = 16\%$	Thr $C_\alpha^0 = 19\%$				
oxa$_m$ $C_0^\alpha = 8\%$ (1.1%)	Asp $C_0^\alpha = 11\%$	Thr $C_0^\alpha = 11\%$		Ile $C_0^\alpha = 11\%$		
oxa$_m$ $C_\beta^\gamma = 15\%$ (1.1%)	Asp $C_\beta^\gamma = 15\%$	Thr $C_\beta^\gamma = 11\%$		Ile $C_{\gamma 1}^\delta = 13\%$		
oxa$_m$ $C_\gamma^\beta = 8\%$ (5%)[2]				Ile $C_\delta^{\gamma 1} = 10\%$		
Pyr$_m$ $C_\alpha^{0'} = 12\%$	Ala $C_\alpha^0 = 7\%$					
Pyr$_m$ $C_\alpha^\beta = 15\%$	Ala $C_\alpha^\beta = 13\%$	Leu $C_\gamma^{\delta 1} = 18\%$		Val $C_\beta^{\gamma 1} = 14\%$		Ile $C_\beta^{\gamma 2} = 17\%$
2 kg$_m$ $C_\alpha^0 = 15\%$	Glu $C_\alpha^0 = 19\%$	Pro $C_\alpha^0 = 20\%$		Arg $C_\alpha^0 = 17\%$		
2 kg$_m$ $C_0^\alpha = 8\%$	Glu $C_0^\alpha = 8\%$					
2 kg$_m$ $C_\alpha^\beta = 19\%$	Glu $C_\alpha^\beta = 23\%$					
2 kg$_m$ $C_\beta^\alpha = 19\%$	Glu $C_\beta^\alpha = 16\%$					
2 kg$_m$ $C_\beta^\gamma = 15\%$	Glu $C_\beta^\gamma = 19\%$	Pro $C_\beta^\gamma = 16\%$		Arg $C_\beta^\gamma = 16\%$		
2 kg$_m$ $C_\gamma^\beta = 28\%$	Glu $C_\gamma^\beta = 28\%$					
2 kg$_m$ $C_\gamma^\delta = 22\%$	Glu $C_\gamma^\delta = 19\%$	Pro $C_\gamma^\delta = 19\%$				
2 kg$_m$ $C_\delta^\gamma = 3\%$	Glu $C_\delta^\gamma = 5\%$	Pro $C_\delta^\gamma = 4\%$		Glu $C_\delta^\gamma = 3\%$		

[1]For abbreviations see table 2.
Numbers in parentheses indicate the conditional enrichments of cytosolic oxaloacetate.

not used for the biosynthesis of Glu in the mitochondria, i.e. Glu is synthesized in the NADH-dependent glutamate synthase reaction (*GLT1*), or NADP-dependent isocitrate dehydrogenase reaction (*IDP1*) is additionally involved in NADPH and Glu synthesis. Although *IDP1* is expressed in mitochondrion, it may not be functional because of the high NADPH/NADP ratio compared to the NADH/NAD, and the reaction of *MAE1* can be the only effective way for NADPH production in the mitochondria during respiratory growth.

It is well established that pyruvate is the precursor for Val and Ala. However, while the synthesis of Val takes place in the mitochondria, some controversy can be found on the compartmental location of the synthesis of Ala. Maaheimo et al. (2001) found in their work with ^{13}C labelled glucose as the sole carbon source that Ala is synthesized from mitochondrial pool of pyruvate (Pyr$_m$) by *ALT1* in *S. cerevisiae*, dos Santos et al. (2003) who used the mixture of labelled acetate and unlabelled glucose have suggested that cytosolic alanine aminotransferase (*ALT2*) is responsible for the amination of cytosolic pyruvate (Pyr$_c$). Our results from the ^{13}C NMR spectra showed that the absolute enrichments of Ala C_0, C_α and C_β were similar to the corresponding carbons of Val and Leu (Table 2), that are both synthesized by the mitochondrial acetolactate synthase (*ILV2* and *ILV6*) (Falco et al. 1985; Pang et al. 1999). This

finding was also strongly confirmed by the fact that similar conditional enrichments in both our experiments were found for Ala $C_{\alpha}{}^{\beta}$, Val $C_{\beta}^{\gamma 1}$, Leu $C_{\gamma}{}^{\delta 1}$ and Ile ${}_{\beta}{}^{\gamma 2}$ representing the same two-carbon fragments in all these amino acids (Table 3). According to Figure 1, the enrichments of the carbons of Pyr_c may not be higher than the natural abundance (1.1%), while the enrichments of the carbons of Pyr_m are higher due to the synthesis through the reaction catalyzed by *MAE1* in the mitochondria (dos Santos et al. 2004). Hence, our data indicate that in the given conditions mitochondrial *ALT1* is functional in the synthesis of Ala.

It has been reported that the carbon skeleton of Asp, Met and Thr is derived from the metabolic pools of cytosolic oxaloacetate (oxa_c) (Michal, 1998) that is synthesized from Pyr_c via the activity of two subunits of enzyme cytosolic pyruvate carboxylase (*PYC1* and *PYC2*) (Kumar et al. 2002). The synthesis of Asp is catalyzed by two isozymes of aspartate aminotransferase (*AAT1* and *AAT2*), the first with the mitochondrial location. Thr and Met are synthesized from Asp over homoserine through the series of reactions catalyzed by the enzymes L-aspartate 4-P transferase (*HOM3*), aspartate semialdehyde degydrogenase (*HOM2*), homoserine dehydrogenase (*HOM6*), homoserine kinase (*THR1*), threonine synthase (*THR4*), homoserine *O*-trans-acetylase (*MET2*), *O*-acetylhomoserine sulfhydrylase (*MET17*) and 5-methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase (*MET6*), which have all been reported to be located in the yeast cytosol (Huh et al. 2003). The ^{13}C NMR spectra of our labelling experiments have, however, shown that the absolute enrichments of C_0, C_{α} and C_{β} of Thr and Asp were remarkably higher than 1.1%, which could have been expected considering the enrichment of C_0,C_{α} and C_{β} of oxa_c, arising from the ^{13}C natural abundance of Pyr_c, as well as of C_{γ} of Thr and Asp that corresponds to C_{γ} of oxa_c the precursor of which is CO_2 (Tables 2 and 3). This was also proved by the absolute enrichments of Ile C_0, C_{α}, $C_{\gamma 1}$ and C_{δ} that stem from C_0, C_{α}, C_{β} and C_{γ} of Thr respectively and was also shown to be of higher absolute enrichment than the natural abundance, and by the calculated conditional enrichments of Thr $C_0{}^{\alpha}$, Asp $C_0{}^{\alpha}$ and Ile $C_0{}^{\alpha}$, or Thr $C_{\beta}{}^{\gamma}$, Asp $C_{\beta}{}^{\gamma}$ and Ile $C_{\gamma 1}{}^{\delta}$ that were remarkably higher than according to the model where oxa_c was considered to be the precursor. Higher absolute enrichment and following conditional enrichment of mitochondrial oxaloacetate (oxa_m) compared to oxa_c can be explained by the higher enrichment of malate C_0, C_{α}, C_{β} and C_{γ} synthesized over the row of consecutive reactions from acetyl-CoA due to the labelled acetate used in our experiments entering the TCA cycle. These results suggest that the synthesis of Asp was carried out by the mitochondrial *AAT1* that was further transported from the mitochondria to the cytosol for the synthesis of Thr, Asn and Met as well as pyrimidines.

Glutamic acid can be synthesized in *S. cerevisiae* either from glutamine and 2-oxoglutarate through the reaction catalyzed by NADH-dependent glutamate synthase (*GLT1*) with the mitochondrial location (Sickmann et al. 2003) or from ammonia and 2-oxoglutarate through the reaction catalyzed by two NADPH-dependent glutamate dehydrogenases (*GDH1,3*) (DeLuna et al. 2001; Magasanik 2003; 2005). The first of the two separate isoenzymes (*GDH1*) is located in the cytosol and the latter (*GDH3*) in the mitochondria (Kumar et al. 2002; Sickmann et al. 2003). There are only few reactions that are involved in NADPH production in *S. cerevisiae* (dos Santos et al. 2004): (i) reactions catalyzed by the two dehydrogenases of PP pathway (*ZWF1* and *GND1,2*); (ii) the reaction catalyzed by NADP-dependent *IDP1*; (iii) the reaction catalyzed by the NADP-dependent acetaldehyde dehydrogenase (*ALD7*); and (iv) reaction catalyzed by *MAE1*. As *ZWF1*, *GND1,2* and *ALD7* are located in the cytosol they are irrelevant with regard to the mitochondrial NADPH production. In contrast to the other mitochondrial isozyme of isocitrate dehydrogenase (*IDH1,2*) the expression of which is low during growth on fermentable carbon sources and is elevated during the growth on non-fermentable carbon sources, *IDP1* has been reported to be incapable of participating in TCA-based respiration despite its mitochondrial location (Haselbeck and McAlister-Henn, 1991) and was thus considered to be negligible with regard to NADPH synthesis during the growth on mixed substrate of acetate and glucose. Taking all this into account, we suppose that in *S. uvarum* in such conditions Glu is synthesized mainly in the reaction catalyzed by NADH-dependent *GLT1* and NADPH required for biosynthetic activity in the mitochondria is derived via the reaction of

MAE1. This hypothesis was supported by the calculation of NADPH requirement in mitochondria for only Val, Ile, Leu and Arg synthesis, i.e. excluding the NADPH requirement for the biosynthesis of other amino acids on the cost of NADPH requirement for the synthesis of Glu. Taking this value equal to the specific flux via *MAE1* in the model (Appendix A) and comparing the experimental absolute enrichments of Ala and Val with those of theoretical calculation for Pyr_m we observed a good fit in both labelling experiments (Tables 2 and 3). On the contrary, if Glu was synthesized mainly through the mitochondrial NADPH-dependent *GDH3* the calculated theoretical absolute enrichments of the carbons of Pyr_m were 2.5 to 3 times higher than the corresponding experimentally obtained enrichments of the carbons in Ala and Val (not shown).

On the basis of theoretical and experimental enrichments we can conclude that the "lower part" of the metabolic scheme of *S. uvarum* generally follows the scheme given on Figure 1. Although the exact determination of the enrinchments in His, Phe, Trp, Tyr, Cys, Gly and Ser could not be carried out, the absolute enrichments in those compounds certainly remained below 2%. The minor incorporation of labelled carbons from ^{13}C-[1,2] Na-acetate into the "upper part intermediates" of cell metabolism was demonstrated in the spectum of trehalose (Figure 2). The low conditional enrichments were noticed in trehalose positions C_γ, C_δ and C_ε ($C_\gamma{}^\varepsilon = 13\%$ and $C_\varepsilon{}^\delta = 16\%$). According to our calculation less than 0.5% of trehalose carbons C_γ, C_δ and C_ε were derived from ^{13}C -[1,2] Na acetate. Further analysis of the possible label movement indicated that the only possible way for the enrichment of trehalose C_γ, C_δ and C_ε could exclusively occur when glyceraldehyde-3-P derived from oxa_m, being enriched in C_0, C_α and C_β as the result of the first steps of glyconeogenesis, reacts with seduheptulose-7-P in PP pathway, resulting in xylulose-5-P and ribose-5-P being enriched in C_β, C_γ and C_δ, which further pass the set of reactions resulting in the enrichment of C_γ, C_δ and C_ε of glucose-6-P. Similarly, Dickinson et al. (1983) have reported that during growth on acetate, glucose synthesized via gluconeogenesis was shunted through the PP pathway. However, somewhat higher enrichments than 1.1% in trehalose can be explained rather by the incorporation of acetate due to the cell cycle or short time oscillations/fluctations of the cell metabolism than by the reversibility of glycolytic reactions.

The NADH-dependent glutamate synthase *(GLT1)* activity has also been demonstrated in insect cells (Drews et al. 2000). Doverskog et al. (2000) have shown that the nitrogen assimilation system glutamine synthetase/glutamate synthase (NADH –GOGAT) is active in glutamine deprived

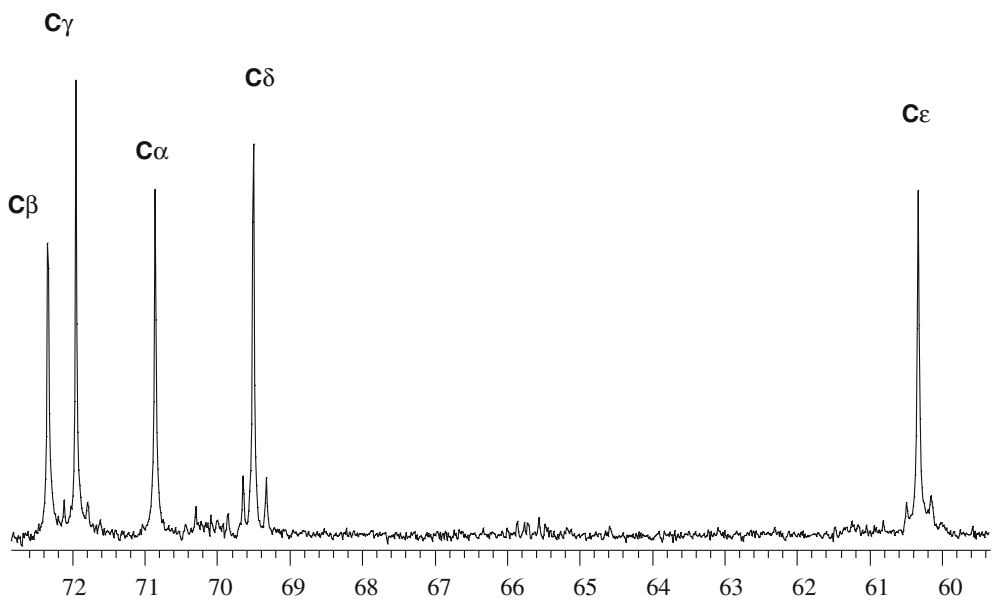

Figure 2. ^{13}C-NMR spectra of trehalose extracted from *S. uvarum* cells grown on glucose and ^{13}C-[1,2] Na-acetate.

Sf9 cells. Our labelling experiments in the Glu free culture media containing Gln have demonstrated that the cells of *S. frugiperda* incorporated the label from ^{13}C-[2] Na-acetate into Ala ($C_0 = 4\%, C_\alpha = 10\%, C_\beta = 10\%$ and $C_\alpha{}^\beta = 23\%$) and Glu($C_\alpha = 4\%$, $C_\beta = 4\%$, $C_\gamma = 12\%$, $C_\beta^\gamma = 20\%$), suggesting that in these conditions (similarly to that of *S. uvarum*) *MAE1* was also involved in the synthesis of Ala. If Ala was synthesised from cytosolic pyruvate derived from glucose, the enrichment would not be expected in case of ^{13}C-[2] Na acetate. Incorporation of the label into Ala can be explained by the synthesis of pyruvate derived from labelled malate in the TCA cycle and further transamination into Ala in the mitochondria of insect cells (Figure 3). It is important to note that in

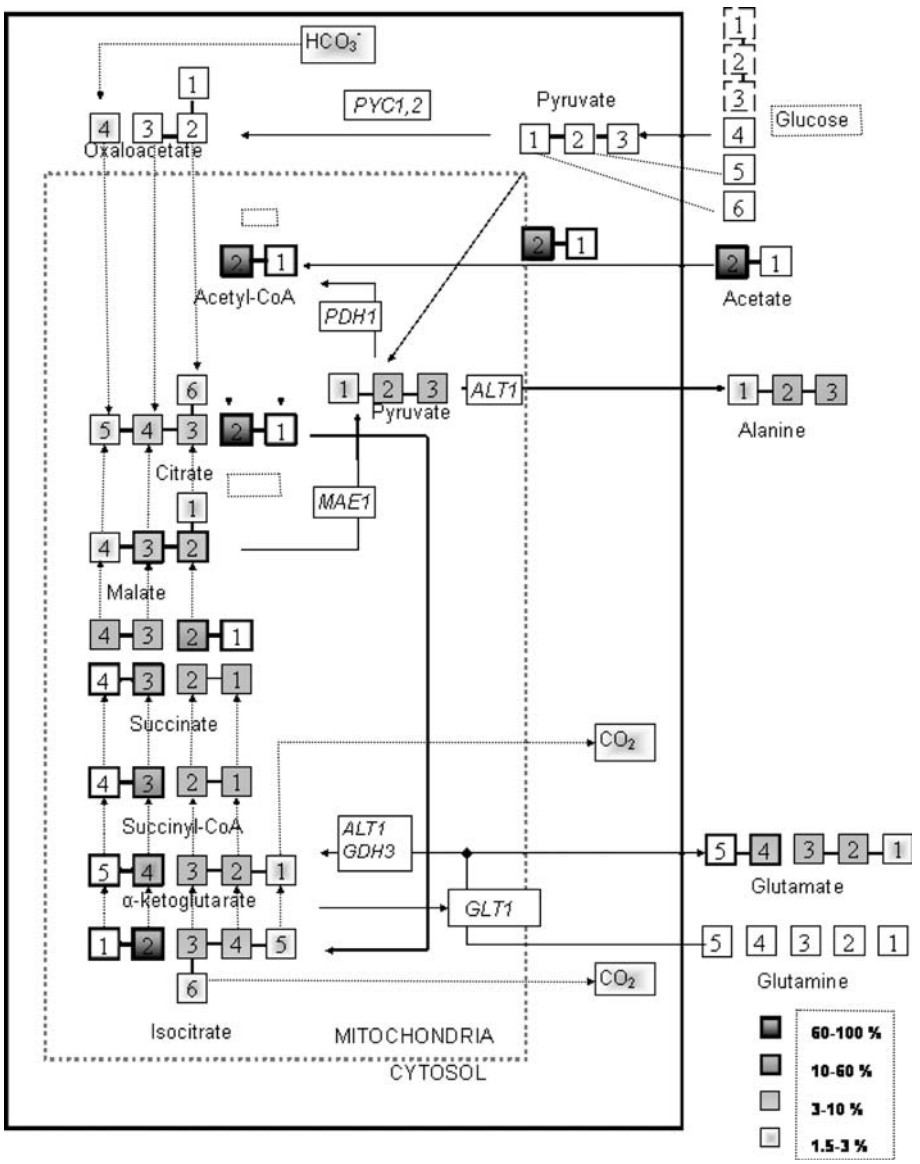

Figure 3. The model of incorporation of the label from ^{13}C-[2] Na-acetate into glutamate and alanine through the TCA cycle of *S. frugiperda*. Key metabolic fluxes are given *in italics: ALT1– alanine transaminase, GDH3 – glutamate dehydrogenase, GLT1 – glutamate syntase, PDH1 – pyruvate dehydrogenase PYC1,2 –pyruvate carboxylase*; movement of the carbons (or carbon fragments) are marked with dashed arrows; values of the absolute enrichments of the carbons are expressed by the different colour intensities.

this case *MAE1* was not required for the synthesis of NADPH as for insect cells Val, Leu and Ile are the essential components of the culture media (Bedard et al. 1993) and thus no mitochondrial NADPH is required for their synthesis. In any case, the role of malic enzyme seems to be the correction of NADPH imbalance in the mitochondria and keeping the NADPH/NADP ratio high enough to support the biosynthetic processes in mitochondrion of both yeast and insect cells.

Conclusions

In contrast to what has been previously described for *S. cerevisiae*, it was found that the subcellular location of Asp and Ala synthesis in *S. uvarum* in given conditions is in the mitochondria. As both the absolute and conditional enrichments of the key metabolic intermediates were very sensitive to the flux values through the malic enzyme which is responsible for the mitochondrial NADPH production in the cells, the best correlation between theoretically calculated and experimentally obtained enrichments were found when the synthesis of Glu was considered through the reaction of NADH-dependent *GLT1*. In general, the ^{13}C NMR metabolic studies on insect cell and yeast culture demonstrated remarkable similarities. Determination of the conditional enrichments of proteinogenic amino acids from the ^{13}C NMR spectra was shown to be an efficient and simple method compared to the determination of absolute enrichments as there is no need for the exact quantification of amino acid concentrations and for the internal standard in the sample.

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Appendix A

Flux equations for the key reactions according to the scheme shown in Figure 1 in *S. uvarum*[1].

Flux (μ mol g^{-1} dwt)	Equation
GND1,2	$GND1,2 = 1/2(-o_{11c} + o_{\mathrm{NADPHc}})$
PYK1,2	$PYK1,2 = 1/6(-12o_1 + o_{11c} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - o_{\mathrm{NADPHc}} + 12q_{glc})$
PYC1,2	$PYC1,2 = \mathrm{MAE1} + o_{10m} + o_{10c} + o_{11c} + o_{11m} + o_{12}$
PC	$PC = 1/6(-6MAE1 - 12o_1 - 6o_{10} - 5o_{11c} - 6o_{11m} - 6o_{12} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - 6o_8 - o_{\mathrm{NADPHc}} + 12q_{glc})$
OAC1	$OAC1 = MAE1 + o_{10m} + o_{10c} + o_{11c} + o_{11m} + o_{12}$
PDH1	$PDH1 = 1/6 - 12o_1 - 6o_{10m} - 6o_{10c} - 5o_{11c} - 6o_{11m} - 6o_{12} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - 6o_8 - o_{\mathrm{NADPHc}} + 12q_{glc}$
CIT1,3	$CIT1,3 = 1/6(-12o_1 - 6o_{10m} - 6o_{10c} - 5o_{11c} - 6o_{11m} - 6o_{12} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - 6o_8 - 6o_{9c} - 6o_{9m} - o_{NADPHc} + 6q_{ace} + 12q_{glc})$
YAT1	$YAT1 = q_{ace} - o_{9c}$
ACO1	$ACO1 = 1/6(-12_{o1} - 6o_{10m} - 6o_{10c} - 11o_{11c} - 6o_{11m} - 6o_{12} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - 6o_8 - 6o_{9c} - 6o_{9m} - o_{NADPHc} + 6q_{ace} + 12q_{glc})$
IDH1,2 = IDP1	$IDH1,2 = IDP1 = 1/6(-12o_1 - 6o_{10m} - 6o_{10c} - 11o_{11c} - 6o_{11m} - 6o_{12} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - 6o_8 - 6o_{9c} - 6o_{9m} - o_{\mathrm{NADPHc}} + 6q_{ace} + 12q_{glc})$
KGD1,2	$KGD1,2 = 1/6(-12o_1 - 6^{o}{}_{10m} - 6o_{10c} - 11o_{11c} - 12o_{11m} - 6o_{12} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - 6o_8 - 6o_{9c} - 6o_{9m} - o_{NADPHc} + 6q_{ace} + 12q_{glc})$
MDH1	$MDH1 = 1/6(-6MAE1 - 12o_1 - 6o_{10m} - 6o_{10c} - 11o_{11c} - 12o_{11m} - 12o_{12} - 12o_2 - 10o_3 - 8o_4 - 6o_5 - 6o_6 - 6o_7 - 6o_8 - 6o_{9c} - 6o_{9m} - o_{\mathrm{NADPHc}} + 6q_{ace} + 12q_{glc})$
MAE1	$MAE1 = o_{\mathrm{NADPH(Ile)}}{}^{2} + o_{\mathrm{NADPH(Val)}}{}^{2} + o_{\mathrm{NADPH(Leu)}}{}^{2} + o_{\mathrm{NADPH(Arg)}}{}^{2}$

[1] For abbreviations of the fluxes see Figure 1.

[2] Requirement of NADPH for the synthesis of Ile, Val, Leu and Arg (calculated based on the concentration in biomass and the requirement of NADPH for the synthesis of 1 μmol of amino acid).

Appendix B

Labeling equations of the key metabolic intermediates according to the model in *S. uvarum*[1].

Intermediate carbon position	Equation
$Pyr_{1(c)}(C_0)$	$Pyr_{1(c)} = 6pg_1 = oxa_{1(c)} = glc_3$
$Pyr_{2(c)}(C_\alpha)$	$Pyr_{2(c)} = 6pg_2 = oxa_{2(c)} = glc_2$
$Pyr_{3(c)}(C_\beta)$	$Pyr_{3(c)} = 6pg_3 = oxa_{3(c)} = glc_1$
$oxa_{4(c)}(C_\gamma)$	$oxa_{4(c)} = CO_2$
CO_2	$CO_2 = -[GND1,2*6pg_1*C + PDH1*pyr_{1(m)}*C + IDP1*icit_{6(m)}*C + mal_4*(KGD1,2*MDH1++MAE1*C)]/(-C*E + KGD1,2*OAC1)$
$Pyr_{1(m)}(C_0)$	$Pyr_{1(m)} = (PC*Pyr_{1(c)} + MAE1*mal_1)/(PC + MAE1)$
$Pyr_{2(m)}(C_\alpha)$	$Pyr_{2(m)} = (PC*Pyr_{2(c)} + MAE1*mal_2)/(PC + MAE1)$
$Pyr_{3(m)}(C_\beta)$	$Pyr_{3(m)} = (PC*Pyr_{3(c)} + MAE1*mal_3)/(PC + MAE1)$
$AceCoA_{1(c)}(C_0)$	$AceCoA_{1(c)} = ace_1$
$AceCoA_{2(c)}(C_\alpha)$	$AceCoA_{2(c)} = ace_2$
$mal_1(C_0)$	$mal_1 = (suc_1 + suc_4)/2 = (2kg_{2(m)} + 2kg_{5(m)})/2$
$mal_2(C_\alpha)$	$mal_2 = D/(2*C*B*A - C*PDH1*MAE1 - B*A*MDH1)$
$mal_3(C_\beta)$	$mal_3 = D/(2*C*B*A - C*PDH1*MAE1 - B*A*MDH1)$
$mal_4(C_\gamma)$	$mal_4 = (2kg_{2(m)} + 2kg_{5(m)})/2$
A	A = YAT1 + PDH1
B	B = PC + MAE1
C	C = OAC1 + MDH1
D	$D = OAC1*oxa_{2(c)}*B*A + C + YAT1*AceCoA_{2(c)}*B + C*PDH1*PC*Pyr_{3(c)}$
E	$E = GND1,2 + PDH1 + IDP1 + KGD1,2 + MAE1$
$oxa_{1(m)}(C_0)$	$oxa_{1(m)} = cit_{6(m)} = (OAC1*oxa_{1(c)} + MDH1*mal_1)/(OAC1 + MDH1)$
$oxa_{2(m)}(C_\alpha)$	$oxa_{2(m)} = 2kg_{3(m)} = (OAC1*oxa_{2(c)} + MDH1*mal_2)/(OAC1 + MDH1)$
$oxa_{3(m)}(C_\beta)$	$oxa_{3(m)} = 2kg_{2(m)} = (OAC1*oxa_{3(c)} + MDH1*mal_3)/(OAC1 + MDH1)$
$oxa_{4(m)}(C_\gamma)$	$oxa_{4(m)} = 2kg_{1(m)} = (OAC1*oxa_{4(c)} + MDH1*mal_4)/(OAC1 + MDH1)$
$AceCoA_{1(m)}(C_0)$	$AceCoA_{1(m)} = 2kg_{5(m)} = (YAT1*AceCoA_{1(c)} + PDH1*Pyr_{2(m)})/(YAT1 + PDH1)$
$AceCoA_{2(m)}(C_\alpha)$	$AceCoA_{2(m)} = 2kg_{4(m)} = (YAT1*AceCoA_{2(c)} + PDH1*Pyr_{3(m)})/(YAT1 + PDH1)$
$Pyr_{12(c)}(C_{0\alpha})$	$Pyr_{12(c)} = oxa_{12(c)} = glc_{23}$
$Pyr_{23(c)}(C_{\alpha\beta})$	$Pyr_{23(c)} = oxa_{23(c)} = glc_{12}$
$oxa_{34(c)}(C_{\beta\gamma})$	$oxa_{34(c)} = CO_2*Pyr_{3(c)}$
$Pyr_{12(m)}(C_{0\alpha})$	$Pyr_{12(m)} = (PC*Pyr_{12(c)} + MAE1*mal_{12})/(PC + MAE1)$
$Pyr_{23(m)}(C_{\alpha\beta})$	$Pyr_{23(m)} = (PC*Pyr_{23(c)} + MAE1*mal_{23})/(PC + MAE1)$
$AceCoA_{12(c)}(C_{0\alpha})$	$AceCoA_{12(c)} = ace_{12} = ace_1/100*ace_2/100$
$mal_{12} = mal_{34}(C_{\beta\gamma})$	$mal_{12} = mal_{34} = (suc_{12} + suc_{34})/2 = (2kg_{12(m)} + 2kg_{45(m)})/2$
$mal_{23}(C_{\alpha\beta})$	$mal_{23} = 2kg_{34(m)} = AceCoA_{2(m)}*oxa_{2(m)}$
$oxa_{12(m)}(C_{0\alpha})$	$oxa_{12(m)} = (OAC1*oxa_{12(c)} + MDH1*mal_{12})/(OAC1 + MDH1)$
$oxa_{23(m)}(C_{\alpha\beta})$	$oxa_{23(m)} = (OAC1*oxa_{23(c)} + MDH1*mal_{23})/(OAC1 + MDH1)$
$oxa_{34(m)}(C_{\beta\gamma})$	$oxa_{34(m)} = 2kg_{12(m)} = (OAC1*oxa_{34(c)} + MDH1*mal_{34})/(OAC1 + MDH1)$
$AceCoA_{12(m)}(C_{0\alpha})$	$AceCoA_{12(m)} = 2kg_{45(m)} = (YAT1*AceCoA_{12(c)} + PDH1*Pyr_{23(m)})/(YAT1 + PDH1)$

[1] Pyr_c and Pyr_m, cytosolic and mitochondrial pyruvate; 6pg, 6-phosphoglycerate; glc, glucose; icit, isocitrate; suc, succinate; mal, malate; oxa_c and oxa_m, cytosolic and mitochondrial oxaloacetate; Ace-CoA_C and Ace-CoA_m, cytosolic and mitochondrial acetyl-CoA; 2 kg_m; mitochondrial 2-ketoglutarate; ace, acetate.

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