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IN VIVO STATIONARY FLUX DETERMINATION USING ^{13}C NMR ISOTOPE LABELLING EXPERIMENTS

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Abstract. The quantitative knowledge of intracellular metabolic fluxes is of fundamental importance for metabolic engineering. Under stationary conditions, standard measurement techniques in combination with NMR labelling data present a rich source of information. A new method is introduced to estimate all intracellular fluxes in central metabolism from this data. It is based on ^{13}C isotope labels from extracted amino acids and a general mathematical model, that explains how those labels are related to fluxes. Moreover the general applicability of the method is supported by automatic generation of the large model equation systems and computer aided flux estimation. An example is given by determining fluxes in *Corynebacterium glutamicum*.

Key Words. Modelling, Parameter estimation, Metabolic engineering, Metabolic fluxes, NMR spectroscopy, Corynebacterium glutamicum, Computer aided data analysis

1. INTRODUCTION

1.1. *Stationary Flux Determination*

One of the most important goals in biotechnology is to develop systematic methods for yield improvement of biosynthetic products (Bailey, 1991). Clearly the *in vivo* determination of intracellular physiological data can be of great profit for any such method. This holds true for genetic manipulations as well as for process and control engineering approaches.

This paper presents a powerful new method for intracellular flux determination under stationary conditions, that combines methods using standard bioprocess measurements (Vallino and Stephanopoulos, 1992) with such based on ^{13}C labels (Malloy *et al.*, 1990). It solely relies on the structure of the metabolic network (see. e.g. Fig. 3) and allows to estimate all intracellular fluxes in central metabolism. I.e. no assumptions about enzyme or transport kinetics have to be made.

The method can be used to characterize different microorganisms under varying physiological conditions. Thereby it will serve as a valuable diagnostic tool to evaluate the success of genetical manipulations. Moreover the knowledge of stationary fluxes is necessary to apply the metabolic design methods proposed by metabolic control theory (Kaczer and Acerenza, 1993). Finally dynamic system models are usually "built around" a stationary state so that they must at least reproduce the stationary flux data.

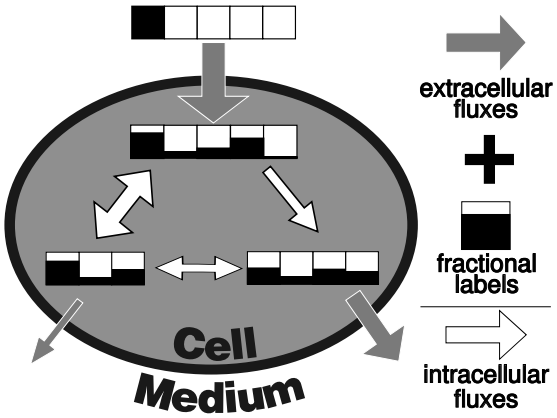

Fig. 1. Types of measurement data used to determine unknown intracellular fluxes: i) extracellular fluxes, ii) fractional labelling of intracellular metabolites.

1.2. *Assumptions on the System*

The sole assumptions made for stationary flux analysis as presented in this paper are:

1. that the observed microbial system is in a well defined stationary physiological state during the measurement procedure. This can be achieved in a controlled continuous culture inside a bioreactor.
2. that for the metabolic pathways of interest, all relevant biochemical transformations are known with respect to i) the involved enzymes, ii) the fate of all carbon atoms within each reaction step and iii) irreversibility in vivo (in so far as this can safely be assumed). For central metabolism this knowledge can be

taken from any biochemistry text book (compare Fig. 3).

3. that enzymes cannot distinguish between different isotopically labelled metabolites with respect to reaction rates. According to current knowledge this is true for liquid phase reaction systems.

These are quite weak assumptions compared with the structural and kinetic assumptions to be made for dynamic system modelling. Therefore flux determination based on these assumptions is expected to produce reliable data sets that are rather unprejudiced with respect to model assumptions.

1.3. *Measurement Data*

To compute all intracellular fluxes for a reasonable complex metabolic network, as much measurement data as possible has to be collected. This must be done with non invasive techniques. Using conventional bioreactor instrumentation, concentrations of substrates and products in the culture medium as well as biomass concentration and gas flows can be measured. From this data the fluxes between the cell cytosol and the surrounding medium can be computed by mass balancing. The resulting fluxes are henceforth called the *extracellular* fluxes (see Fig. 1).
The number of significant extracellular fluxes is usually not more than 10 (growth rate, substrate uptake, product formation, O_2 uptake, CO_2 formation, some byproducts, ammonium uptake). A method for flux quantification in a complex metabolic network that is solely based on this data set was proposed in (Vallino and Stephanopoulos, 1992). However it turned out that some additional assumptions are required to get a well determined equation system for flux estimation from so few data. In practice several enzymes were considered inactive and the thermodynamic efficiency of ATP generation from NADH was assumed.
The new approach for flux determination uses NMR measurements of ^{13}C isotope labels *in addition* to the standard extracellular flux data. Thereby the amount of available system information is significantly increased. Moreover it is possible to determine not only net fluxes but also exchange rates of reversible reactions (as will be shown in section 3). The method is described in the following sections together with an application to *Corynebacterium glutamicum*.

2. EXPERIMENTAL SETUP

This section gives a short sketch of the experimental methods used to obtain the required measurement data. More details will be published elsewhere (Marx *et al.*, 1995).

To achieve a well defined stationary physiological state, cells are grown in continuous culture. Because labelled substrates are extremely expensive, a small bioreactor with volume of 325 ml is used. All relevant extracellular metabolite concentrations as well as gas flows are measured by using standard instrumentation.
After reaching the physiological steady state, the substrate is replaced by ^{13}C labelled substrate. From this moment on the ^{13}C atoms become distributed all over the metabolic network until an isotopic steady state is reached. However, most intracellular intermediates are too weakly concentrated to become accurately detectable by NMR. Therefore an alternative method to get labelling data is used. It relies on the fact that metabolic intermediates gradually become incorporated into biomass with their labelling state remaining conserved. When about 5 fermentor residence times have been passed, about 99% of the biomass has been exchanged by labelled material. This is the time for harvesting.
A rich source of differently labelled cell components is presented by the cell proteins, that are therefore extracted and hydrolized. The resulting amino acids are then purified preparatively by FPLC. A small amount of such a concentrated amino acid is sufficient to produce an NMR signal. A close look to the biosynthesis of amino acids shows, that most of them stem directly from precursors in central metabolism. Thereby the *in vivo* fractional labelling in many important intermediates of central metabolism can be reconstructed.

3. A SIMPLE EXAMPLE

The principles of intracellular flux estimation from the obtained ^{13}C labels combined with the measured extracellular fluxes are explained now. Before formulating a general (but rather abstract) mathematical model, the equations will be introduced for the case of a very simple example network. The model equations presented in the following are well known from tracer kinetics (Anderson, 1983). However they are adapted here to the special structure of metabolic networks.

3.1. *Example Network*

The network shown in Fig. 2 was designed to demonstrate the effect of reversible reactions often occurring *in vivo*. As is shown below, both directions of a reversible reaction step can influence the label distribution. Although being oversimplified, the example illustrates that complex reaction systems (like the pentose phosphate pathway) — including exchanging steps — can be quantitated from measured labels.

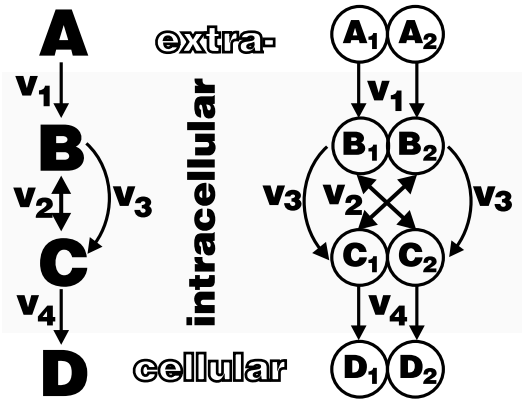

Fig. 2. A simple example network. Left: metabolite view. Right: carbon atom view. The extracellular flux v_1 and the labels B_1, C_1 are assumed to be measurable.

The substrate uptake v_1 as well as the fractional labels in carbon atoms B_1 and C_1 are assumed to be measurable. The intracellular flux v_2 takes place in both directions while the others (v_1, v_3, v_4) are irreversible.

3.2. *State Variables*

The system will be described in terms of fluxes v_i and labels x_j. Both flux directions of a reversible reaction step must be accounted for. In the example the forward and backward flux of v_2 are represented by the symbols $v_2^{\rightarrow}, v_2^{\leftarrow}$. The corresponding net flux is then given by $v_2^{\text{net}} = v_2^{\rightarrow} - v_2^{\leftarrow}$. The other flux variables are simply denoted by v_1, v_3, v_4 (instead of $v_1^{\rightarrow}, v_3^{\rightarrow}, v_4^{\rightarrow}$).
The fractional labels at each carbon atom position of intracellular metabolites are denoted by $B_1, B_2, C_1, C_2 \in [0, 1]$. Additionally the known input to the system is specified by A_1, A_2. E.g. a substrate A which is 100% labelled at the first carbon atom position, is described by $A_1 = 1, A_2 = 0$. The terminal nodes D_1, D_2 are not required.

3.3. *Flux and Label Balances*

The metabolite net fluxes from and to an intracellular metabolite pool must sum up to zero in stationary state. This gives rise to the following linear balance equations at stage B and C:

$$\begin{array}{rcl} v_1 & = & v_2^{\text{net}} + v_3 \\ v_4 & = & v_2^{\text{net}} + v_3 \end{array} \qquad (1)$$

from which follows

$$v_4 = v_1 \quad \text{and} \quad v_3 = v_1 - v_2^{\text{net}} \ . \qquad (2)$$

This leaves $v_2^{\leftarrow}, v_2^{\rightarrow}$ to be determined (remember that v_1 is measured directly).
The carbon balance equations are established now. To this end it must be recognized that a flux variable v_i has two meanings: It denotes a molecular flux (with unit [mol/h]) as well as a carbon flux (with unit [C-mol/h]). However, the values of both fluxes are identical. For this reason it is not distinguished between both meanings. Having this in mind, the flux $v_3 : B_1 \rightarrow C_1$ (interpreted as a carbon flux rather than a molecular flux) carries the amount of $v_3 \cdot B_1$ labelled carbon atoms per time unit. This term contributes to the label balances of B_1 and C_1, but with opposite signs. The complete set of equations for the pools B_1, B_2, C_1, C_2 then is:

$$\begin{array}{rcl} 0 & = & v_1\ A_1 - v_2^{\rightarrow} B_1 + v_2^{\leftarrow} C_2 - v_3 B_1 \\ 0 & = & v_1\ A_2 - v_2^{\rightarrow} B_2 + v_2^{\leftarrow} C_1 - v_3 B_2 \\ 0 & = & v_2^{\rightarrow} B_2 - v_2^{\leftarrow} C_1 + v_3\ B_1 - v_4 C_1 \\ 0 & = & v_2^{\rightarrow} B_1 - v_2^{\leftarrow} C_2 + v_3\ B_2 - v_4 C_2 \end{array} \qquad (3)$$

3.4. *Flux Determination*

In the example the flux determination problem can be solved explicitly. We are finished when $v_2^{\rightarrow}, v_2^{\leftarrow}$ are computed from v_1, B_1, C_1. Firstly the label conservation equations

$$A_1 + A_2 = B_1 + B_2 = C_1 + C_2$$

can be derived from (3). Using this, the unknown fluxes are calculated to be:

$$\begin{array}{rcl} v_2^{\leftarrow} & = & \frac{B_1 - A_1}{A_1 + A_2 - B_1 - C_1} \cdot \qquad v_1 \\ v_2^{\rightarrow} & = & \frac{C_1 - B_1}{A_1 + A_2 - 2B_1} \ \cdot (v_2^{\leftarrow} + v_1) \end{array} \ .$$

This shows how the rates of reversible reactions can be computed from labelling data. With conventional measurement techniques only the corresponding net flux rates could be observed!

4. GENERAL FLUX MODEL

A general model for arbitrary metabolic networks is formulated now. Based on this model, fractional labels can be computed when all fluxes are known. The inverse problem of flux estimation from known labelling data can then be solved via parameter fitting.
The model makes extensive use of matrix calculus to obtain a concise notation. All matrices are more or less sparsely populated which can be taken into account for their numerical implementation. It is aimed at a *structural* matrix representation of the metabolic system. This means that all matrices are constant and represent structural qualities of the underlying network, i.e. they do not depend on the state variables. Structural representations are desirable for further mathematical systems analysis.

4.1. *State Variables*

The vectors

$$\mathbf{v}^{\rightarrow}, \quad \mathbf{v}^{\leftarrow}$$

(of equal dimension) comprise both directions of all molar fluxes in the reaction network. In the example of section 3 these fluxes are $\mathbf{v}^{\rightarrow} = (v_1, v_2^{\rightarrow}, v_3, v_4)^T$ and $\mathbf{v}^{\leftarrow} = (0, v_2^{\leftarrow}, 0, 0)^T$ (the zeroes represent the irreversibility assumptions made in the example). From these vectors the composite overall flux vector (of twice the dimension) and the corresponding net flux vector is formed:

$$\mathbf{v} = \begin{pmatrix} \mathbf{v}^{\rightarrow} \\ \mathbf{v}^{\leftarrow} \end{pmatrix}, \quad \mathbf{v}^{\text{net}} = \mathbf{v}^{\rightarrow} - \mathbf{v}^{\leftarrow} . \tag{4}$$

Later on a measure for expressing the exchange rate of reversible reactions is needed. This is defined to be the amount of flux going in both directions relative to the net flux:

$$\mathbf{v}_i^{\leftrightarrows} = \min(\mathbf{v}_i^{\rightarrow}, \mathbf{v}_i^{\leftarrow}) / \mathbf{v}_i^{\text{net}} \tag{5}$$

Finally the vector of intracellular fractional labels of each (enumerated) carbon atom in the intracellular metabolites, as well as the vector of input labels comprising the known fractional labels of all carbon atoms, that are fed into the system, are required. These two vectors are denoted by

$$\mathbf{x}, \quad \mathbf{x}^{\text{inp}} .$$

In the example $\mathbf{x} = (B_1, B_2, C_1, C_2)^T$ and $\mathbf{x}^{\text{inp}} = (A_1, A_2)^T$.

4.2. *Flux and Label Balances*

The flux balances are formulated by using the well known stoichiometric matrix $\mathbf{N}$ and equation (4) which leads to the linear equation system

$$\mathbf{N} \cdot \mathbf{v}_{net} = \mathbf{N} \cdot \mathbf{v}^{\rightarrow} - \mathbf{N} \cdot \mathbf{v}^{\leftarrow} = \mathbf{0} . \tag{6}$$

In the example (1)

$$\mathbf{N} = \begin{pmatrix} 1 & -1 & -1 & . \\ . & 1 & 1 & -1 \end{pmatrix} .$$

The label balance equations (3) have a bilinear structure with respect to $\mathbf{x}$ and $\mathbf{v}$. All terms containing a certain flux $\mathbf{v}_i$ are of type $\pm x_j v_i$ and contribute to some pool x_k. The coefficients ± 1 can therefore be collected in a square Matrix P_i and a rectangular Matrix P_i^{inp} associated with v_i. P_i belongs to transitions between intracellular pools, while P_i^{inp} collects coefficients with one extracellular partner. In the example (3):

$$\mathbf{P}_2^{\rightarrow} = \begin{pmatrix} -1 & . & . & . \\ . & -1 & . & . \\ . & 1 & . & . \\ 1 & . & . & . \end{pmatrix}, \mathbf{P}_1^{\rightarrow,\text{inp}} = \begin{pmatrix} 1 & . \\ . & 1 \\ . & . \\ . & . \end{pmatrix}$$

Using matrix notation the carbon pool balance equations can now be written as:

$$(\sum_i \mathbf{v}_i \cdot \mathbf{P}_i) \cdot \mathbf{x} + (\sum_i \mathbf{v}_i \cdot \mathbf{P}_i^{\text{inp}}) \cdot \mathbf{x}^{\text{inp}} = \mathbf{0} . \tag{7}$$

From these equations follows immediately that

$$\begin{aligned} \mathbf{x} &= \mathbf{x}(\mathbf{v}) \\ &= -(\textstyle\sum_i \mathbf{v}_i \cdot \mathbf{P}_i)^{-1} (\sum_i \mathbf{v}_i \cdot \mathbf{P}_i^{\text{inp}}) \cdot \mathbf{x}^{\text{inp}} \end{aligned} \tag{8}$$

is a function of $\mathbf{v}$. This equation is the basis for the simulation of ^{13}C tracer experiments. It can be solved by matrix factorization or iterative methods. The latter is promoted by the sparse matrix structure (Anderson, 1983).

4.3. *Measurement Equations*

The measurement matrices $\mathbf{M_V}, \mathbf{M_X}$ are used to describe which net fluxes resp. intracellular labels are measured. They are both composed from unit vectors. In the example v_1, B_1, C_1 are measured and therefore we have:

$$\mathbf{M_V} = \begin{pmatrix} 1 & . & . & . \end{pmatrix}, \quad \mathbf{M_X} = \begin{pmatrix} 1 & . & . & . \\ . & . & 1 & . \end{pmatrix}$$

The complete measurement equations then are:

$$\begin{aligned} \mathbf{w} &= \mathbf{M_V} \cdot \mathbf{v}^{\text{net}} + \varepsilon_{\mathbf{V}} \\ &= \mathbf{M_V} \cdot (\mathbf{v}^{\rightarrow} - \mathbf{v}^{\leftarrow}) + \varepsilon_{\mathbf{V}} \\ \mathbf{y} &= \mathbf{M_X} \cdot \mathbf{x} + \varepsilon_{\mathbf{X}} \end{aligned} \tag{9}$$

Herein $\mathbf{w}, \mathbf{y}$ denote the vectors of measured fluxes resp. labels, $\varepsilon_{\mathbf{V}}, \varepsilon_{\mathbf{X}}$ are normally distributed measurement noise terms with expectation $\mathbf{0}$ and covariance matrices $\mathbf{\Sigma_V}, \mathbf{\Sigma_X}$.

4.4. *Flux Estimation*

Unlike in the example the general flux determination problem cannot be solved explicitly. It has to be done by parameter fitting. The familiar least squares flux estimator is constructed from (9) by minimizing the sum of squares function

$$\begin{aligned} \kappa(v) = &||\mathbf{w} - \mathbf{M_V} \cdot (\mathbf{v}^{\rightarrow} - \mathbf{v}^{\leftarrow})||^2_{\mathbf{\Sigma_V}} \\ &+ ||\mathbf{y} - \mathbf{M_X} \cdot \mathbf{x}(\mathbf{v})||^2_{\mathbf{\Sigma_X}} \end{aligned} \tag{10}$$

where $||\xi||^2_{\mathbf{\Sigma}} = \xi^T \cdot \mathbf{\Sigma}^{-1} \cdot \xi$ denotes the squared weighted norm corresponding to a covariance matrix $\mathbf{\Sigma}$. Putting equations (6), (8) and (9) together we are left with a linearly constrained minimiza-

tion problem for flux estimation:

$$\hat{\mathbf{v}} = \underset{\mathbf{N}\cdot(\mathbf{v}^{\rightarrow}-\mathbf{v}^{\leftarrow})=\mathbf{0}}{\arg\min} \kappa(\mathbf{v}) \quad . \tag{11}$$

4.5. *Numerical Solution*

From the linear constraints (6) several flux variables can be eliminated as has been done in (2). In a numerically stable way this can be done by using singular value decomposition. The unconstrained minimization problem resulting thereby from (11) can then is solved with an iterative optimization method.

4.6. *Additional Constraints*

When all fluxes in the overall flux vector $\mathbf{v}$ have to be estimated, the number of measurements that are required to determine the whole system might be too high. Fortunately, if the exchange between two pools is assumed to be very high compared to the net flux rate, several variables can be eliminated by pool lumping (Schuster *et al.*, 1992). Moreover many reaction steps (i.e. extracellular fluxes) can or must be considered to be irreversible from thermodynamic considerations. Thereby the a priori knowledge about the biochemical system can be used to further reduce the number of independent parameters.

5. COMPUTER AIDED DATA ANALYSIS

All model equations required to describe the isotope isomer labelling system are built up from quite large vectors and matrices. A manual input of these structures would be tedious and susceptible to errors. Moreover model variation studies would be quite long-winded when using manual input. A program was written therefore that automatically generates all required structures from a familiar textual description of the biochemical network together with the measured data. E.g. the the transaldolase-reaction from the phosphate pathway is written as

```
TA : GA3P + S7P       > E4P   + F6P
     #ABC + #abcdefg > #defg + #abcABC ;
```

This means that the first carbon atom of `GA3P` (denoted `A`) is taken over to the fourth carbon atom of `F6P` and so on. The program uses the well known recursive descent parsing algorithm for translating the equations. See Wiechert (1994*b*) for more details.
Another program takes the generated vectors and matrices as input and performs the numerical computations as described before. All flux parameters can be changed interactively to do simulation studies. Finally a minimization procedure can be set up. All results are written to a protocol file for further use. More details can be found in (Wiechert, 1994*a*).

6. APPLICATION TO C. GLUTAMICUM

The procedure described in section 2 was carried out with lysine producing *Corynebacterium glutamicum* MH20-22B. Cells were harvested after 30h incubation with 1-^{13}C-glucose. Figure 3 shows the biochemical network that was taken as structural input for flux determination. Since the exact biomass composition is known, the measurable overall flux into biomass splits up into several fluxes of cell components. Table 2 summarizes the measured values for amino acid labels while the measured extracellular fluxes can be taken from Fig. 3. Based on this structural and measurement data, all intracellular fluxes together with several exchange rates of reversible reactions could be determined. The results are summarized in Table 1. As can be further seen from Table 2, the measured labels have been reproduced quite good by the model. The same holds true for the measured fluxes with an even higher precision (not shown here). A detailed discussion of the experimental conditions and the biochemical implications of the obtained results is is given in Marx *et.al.* (1995).

7. CONCLUSION

A method for stationary intracellular flux determination has been developed, that proved to be well applicable to microorganisms. Because of its generality and an automated process of data analysis it can be quickly adapted to arbitrary metabolic networks. The usage of amino acids proved to open a rich source of meaningful labelling data. The experimental techniques are presently being accelerated and standardized to achieve a routine procedure for variational studies. Further work must be done to improve the parameter fitting process by incorporating further (i.e. nonlinear) constraints. To this end a nonlinear equality constrained optimization algorithm of Marquardt type is currently being implemented. Valuable insights concerning identifiability, redundancy and sensitivity have been obtained recently. They will be published elsewhere. Finally the planning of labelling experiments can be further facilitated by applying graph algorithms as described in Wiechert (1994*b*).

Table 1
Estimated intracellular net fluxes and exchange rates that could be identified.

Flux	Est.	Exch.
f_1	67.0	
f_2	28.9	
f_3	66.4	
f_4	1.6	
v_0	31.6	8.9
v_1	71.3	
v_2	40.2	1.1
v_3	26.9	0.0
v_4	21.0	31.2
v_5	21.0	1.6
v_6	19.2	0.0
v_7	71.3	0.0
v_8	161.1	108.9
v_9	177.6	
v_{10}	63.5	
v_{11}	61.9	
v_{12}	53.6	
v_{13}	55.3	2.3
v_{14}	55.9	0.0
v_{15}	1.0	

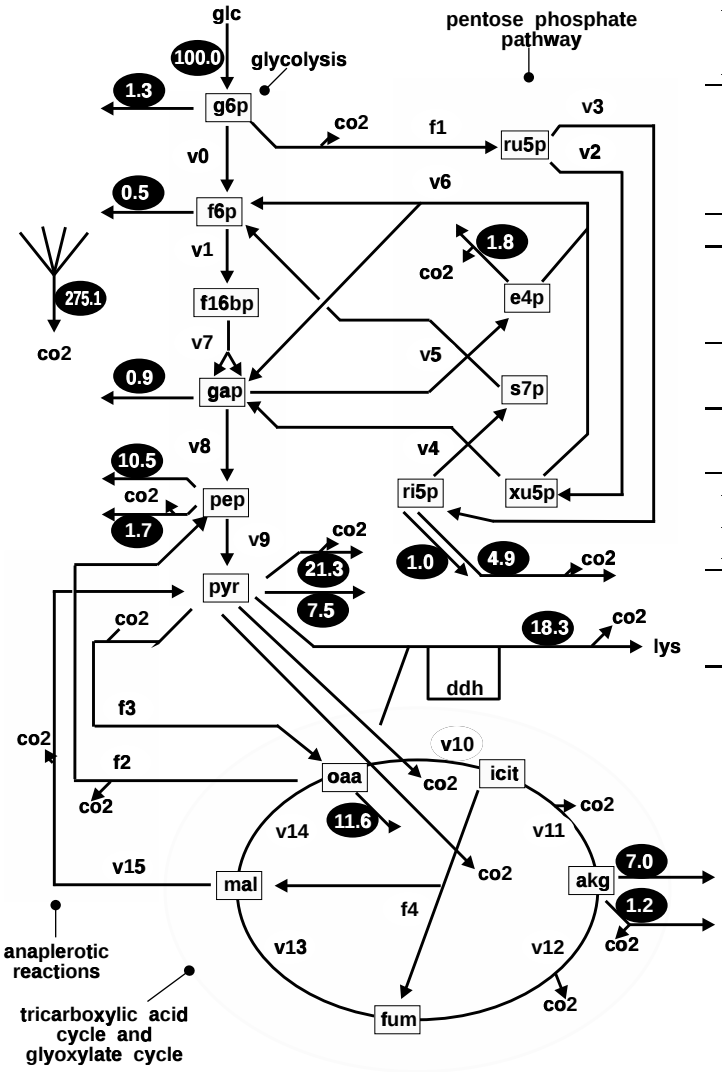

Table 2
Some measured labels compared to values predicted by the model.

Atom	Meas.	Est.
$E4P_1$	2.0	2.5
$E4P_2$	3.6	2.0
$E4P_3$	2.0	1.9
$E4P_4$	16.7	15.3
CO2	23.0	21.6
GAP_1	2.9	2.7
GAP_2	2.6	2.6
GAP_3	26.7	26.3
PEP_2	2.5	2.6
PEP_3	26.5	26.3
PYR_2	3.0	2.7
PYR_3	26.4	26.3
AKG_2	24.1	22.6
AKG_3	11.1	9.8
AKG_4	28.1	26.3
OAA_2	7.6	9.8
OAA_3	20.9	22.6
OAA_4	16.8	17.3
LYS_2	6.8	7.1
LYS_3	21.9	24.0
LYS_4	18.9	17.3
LYS_5	22.2	24.9
LYS_6	5.6	5.3

Fig. 3. Structure of metabolic network (central metabolism) as used for *C. glutamicum*. The shaded regions comprise intracellular intermediates in certain pathways. The measured extracellular fluxes (including fluxes into biomass) are given in circles. Intracellular fluxes are denoted by v_i, f_i.

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