Error analysis of metabolic-rate measurements in mammalian-cell culture by carbon and nitrogen balances

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Abstract

The analysis of metabolic fluxes of large stoichiometric systems is sensitive to measurement errors in metabolic uptake and production rates. It is therefore desirable to independently test the consistency of measurement data, which is possible if at least two elemental balances can be closed. For mammalian-cell culture, closing the C balance has been hampered by problems in measuring the carbon-dioxide production rate. Here, it is shown for various sets of measurement data that the C balance can be closed by applying a method to correct for the bicarbonate buffer in the culture medium. The measurement data are subsequently subject to measurement-error analysis on the basis of the C and N balances. It is shown at 90% reliability that no gross measurement errors are present, neither in the measured production- and consumption rates, nor in the estimated in- and outgoing metabolic rates of the subnetwork, that contains the glycolysis, the pentose-phosphate, and the glutaminolysis pathways.

Introduction

Intracellular metabolic fluxes can be determined from extracellular uptake and secretion rates of relevant metabolites using mass-balancing techniques (Vallino and Stephanopoulos, 1989; Varma and Palsson, 1994). A major problem of this method which applies particularly for large, complex networks is that the estimated fluxes are sensitive to errors in the measured extracellular rates. It is therefore desirable to test measurement data on possible errors independent of the flux-balance equations (Noorman et al., 1991). Alternative conservation equations that are independent from flux balances can be derived from heat or elemental balances.

Elemental balances have been used to test fermentation data for prokaryotes and yeast (Roels, 1983; Ferrer and Erickson, 1980; Wang and Stephanopoulos, 1983; Noorman et al., 1991). For mammalian-cell

culture however, an error analysis based on elemental balances is hampered by the fact that relatively few balances can be closed. In contrast to fermentations of yeast or prokaryotes (Von Stockar et al., 1997), the heat balance cannot be used because mammalian cells generate only a fraction of the energy that is required to control the cell-culture bioreactor at the desired temperature. The elemental balances for hydrogen and oxygen can not be closed, since (mammalian) cells secrete or take up water at negligible rates compared to the water balance of the bioreactor system. In addition, it is not possible to quantify the H⁺-production rate at sufficient accuracy for error analysis due to the presence of buffer systems. Therefore, only the nitrogen and carbon mass balance are suitable to trace measurement errors in mammalian-cell culture.

A prerequisite to close the carbon balance is the correct measurement of the carbon-dioxide evolution rate. In mammalian-cell culture this is hampered by

the use of a bicarbonate-based buffer system in the culture medium. Methods to determine the carbon-dioxide production rate of mammalian cells cultured in bicarbonate-buffered media have recently been described for continuous (Bonarius et al., 1995) and batch (Zupke and Stephanopoulos, 1995) cultures. Here, it is shown that when such methods are used the carbon balance of mammalian-cell culture can be closed within an acceptable probability range. Together with the nitrogen balance, the two constraints that are determined by these elemental balances theoretically allow the detection of gross measurement errors within the set of measured uptake and production rates. Here, the method to detect gross measurement errors, described by Ripps (1965) and developed by Wang and Stephanopoulos (1983) for data from microbial metabolism, is applied for mammalian-cell culture under 7 different culture conditions. Various sets of measurements that are used to estimate the intracellular fluxes are subjected to this particular test.

Materials and methods

Culture conditions and analyses

A detailed description of the various experimental procedures was published before (Bonarius et al., 1996). Briefly, hybridoma cells were cultured in lab-scale bioreactor (B. Braun Diessel Biotech, Melsungen, Germany) in a continuous mode at a dilution rate of 0.7 d^{-1} . The fraction of CO_2 in the outlet gas was measured using an infrared gas analyzer (Rosemount, Baar, Switzerland). Glucose and lactate were determined with automated enzymatic assays, ammonia using an ion-selective electrode, and amino acids by HPLC (Amino Quant 1090, Hewlett-Packard, Palo Alto, CA). The cellular composition was measured as follows (Xie and Wang, 1994): the total lipid fraction was determined by weight after chloroform/methanol extraction, total carbohydrates were analyzed by the phenol-reaction method, and total cellular protein was estimated using the Biuret assay. Cell size and number were determined using a Casy 1 instrument (Schärfe, System, Reutlingen, Germany) and dry-cell weight was determined after dehydration under vacuum. Antibody titers were measured by a standard ELISA. Free intracellular amino-acid pools were extracted by perchloric acid as described by Schmid and Keller (1992). After neutralization and lyophilization, the fraction of the free amino acids of the intracellular pools were quantified by HPLC. The values for

intracellular protein-bound amino acids were taken from measurements of hydrolyzed protein (1 N HCl, 125°C , overnight) of the same cell line, grown under the same medium conditions (Bonarius et al., 1996).

Statistical analysis

From the law of mass conservation it follows that when all (produced and consumed) metabolites that significantly contribute to the total mass of an element are determined, the residual value of the elemental balance (ε) can be used as the estimated measurement error for the elemental balance. Wang and Stephanopoulos (1983) used a statistical test function to judge whether the residual values deviate significantly away from their expected distribution of zero means. In this function, h_ε , the residuals are weighted according to their accuracy (or standard deviation of the measured production and consumption rates). In the case of two elemental balances (here, carbon and nitrogen), a lower value of h_ε than 4.61 indicates with a confidence level of 90% that no gross measurement errors are presented. The value of 4.61 for h_ε has been justified by Wang and Stephanopoulos (1983) for all microbiological systems: If the residuals (ε) are assumed to have identical and independent (ϕ , the variance-covariance matrix for ε , is diagonal) normal distribution around 0, then the test function h_ε ($= \varepsilon^T \phi^{-1} \varepsilon$) follows a χ^2 -distribution with m degrees of freedom, where m is the number of constraint equations.

In this work, each data point is an average of a double measurement taken at three time points during each steady state (the last three days of each condition). Here, a 'steady state' is obtained when the difference in viable cell density from the average value is smaller than 10%. Variances of all measured metabolites are estimated from these six experimental data points. An exception is the total-lipid assay which was only carried out once for each set of measurements. This assay requires relatively large amounts of cells because it is a weight measurement. The sampling of large volumes disturbs the steady-state conditions. The variance of the lipid measurement is therefore not estimated from experimental data, but assumed to be 10% of the measured value. Further, due to lack of material from the second, third, and fourth steady state, the average value of the lipid and the DNA measurement of the first and fifth steady state was used for the first five steady states (Table 4a).

Figure 1. Network of mammalian-cell metabolism. Fluxes that can be quantified by mass balances alone are indicated as dashed lines. To measure the remaining fluxes (solid lines) additional constraints are required. The two networks of which the measured in- and outgoing metabolic rates are subject to error analysis, are shown: (i) the entire metabolic network (dotted line) and (ii) the underdetermined sub-network (L-shaped box). Abbreviations: ACoA acetyl-CoA, AKG α -ketoglutarate, CHOL cholesterol, CIT citrate, E4P erythrose-4-phosphate, GAP glyceraldehyde 3-phosphate, GLC glucose, G6P glucose-6-phosphate, 3PG 3-Phosphoglycerate, LAC lactate, MAL malate, OAA plus oxaloacetate, PEP phosphoenolpyruvate, PYR pyruvate, R5P ribose-5-phosphate, Ru5P ribulose-5-phosphate, S7P sedoheptulose-7-phosphate, TC total carbohydrates. TP total protein, X5P xylulose-5-phosphate. The complete stoichiometry of the reaction network, which includes the by-products and co-metabolites of the given reactions, is given elsewhere (Bonarius et al., 1996).

Similarly to Wang and Stephanopoulos (1983), it is assumed that the errors are uncorrelated. In reality some correlation in measurements are unavoidable. For instance, the amino-acid measurements may be correlated because all amino-acid concentrations are determined simultaneously by HPLC. Errors that occur in dilution of samples for HPLC or calibration errors are correlated. However, when (non-negative) co-variances are taken into account the test function h_ε will decrease. So, when it is assumed that the measurements are not correlated, the upper limit of h_ε is determined, which implies that for a value of 4.61 the confidence level is equal or greater than 90%.

Certain fluxes, in particular fluxes in cyclic pathways, cannot be determined by mass-balancing techniques alone, because their reaction stoichiometry is linear dependent (Vallino and Stephanopoulos, 1989; Bonarius et al., 1997). In the metabolic network that describes mammalian-cell metabolism, a subsystem remains therefore underdetermined (See also Figure 1). The fluxes of this subnetwork need to be quantified

by isotopic-tracer experiments. If this is not possible, which is for example the case for large-scale cell culture, the fluxes of this smaller network can only be estimated using additional assumptions. In order to prevent that such assumptions affect the determinable fluxes (dashed lines in Figure 1), the non-determinable fluxes (solid lines in Figure 1) are estimated separately as described elsewhere (Bonarius et al., 1998a, 1998b). Here both metabolic systems are subject to gross measurement error analysis:

- (i) the entire metabolic network, and
- (ii) the underdetermined subnetwork.

Results and discussion

Hybridoma cells were cultivated in a continuous stirred-tank reactor under different conditions. The pO_2 was varied and in another experiment PMS was added to the culture medium. Viable-cell density, viability, and dilution rate are shown in Figures 2a and 2b. The extracellular production and consumption rates of amino acids, glucose, ammonia, lactate, carbon dioxide and monoclonal antibody were determined. Also, the intracellular concentrations of amino acids, lactate, total cell protein, DNA, RNA, and the total lipid and carbohydrate contents were measured. After changing to new parameter settings at least four days of continuous culture were used to dilute remaining metabolites during the previous steady state and to allow the viable cell density to stabilize. Steady-state conditions were obtained in all experiments, except during the oxygen limitation experiment ("00" in Figure 2a) in which 'pseudo steady-state' conditions were obtained.

Table 2 and 3 show the average measured metabolic rates and their standard deviations for the entire network (i) and the smaller, underdetermined network (ii), respectively. The average viable-cell numbers and implications of these results for the cell physiology and metabolism of hybridoma cells are reported elsewhere (Bonarius et al., 1998b).

In Table 4 the residual values for carbon and nitrogen are given for the various sets of measured uptake and production rates (in 10^{-12} C-mol.cell $^{-1}$ and 10^{-12} N-mol.cell $^{-1}$. day $^{-1}$, respectively). A positive value indicates either an overestimation of the produced carbon or nitrogen, or an underestimation of the consumed carbon or nitrogen. The total turnover rate (the sum of all produced and consumed metabolites) is shown for comparison. In addition, the relative

Table 1. Elemental composition of measured macromolecules

	C	N	Source
Protein			
Measured composition (w/Prim. ^a)	4.879	2.475	Bonarius et al., 1996
Composition literature	4.743	2.411	Creighton et al., 1984
Composition Mabs literature	4.840	2.378	Edelman et al., 1964
RNA/DNA	9.50	3.75	Sreyer, 1988
Lipids (w/Prim. ^a)	18.45	0	Bonarius et al., 1996

^a “w/Prim.” refers to measured amino acids in cellular total protein from cells cultured with Primatone RL in the medium.

Table 2a. Average measured metabolic rates of metabolites of entire metabolic network for seven steady states (Values in 10^{-12} mol. cell⁻¹. day⁻¹. Abbreviations as in Figure 1. Amino acids are given with the standard three-letter codes.

	PO2 30	PO2 50	pO2 1	pO2 0	pO2 100	pO2 30	PMS
ASP	-0.088	-0.06	-0.044	-0.002	-0.078	-0.173	-0.078
GLU	-0.071	-0.027	-0.054	0.011	-0.029	-0.208	-0.074
ASN	-0.061	-0.068	-0.055	-0.037	-0.047	-0.013	-0.093
SER	0.041	-0.025	-0.113	-0.097	0.023	-0.023	0.080
GLN	-2.102	-1.618	-1.442	-2.025	-1.941	-1.823	-2.140
HIS	-0.072	-0.058	-0.047	-0.054	-0.068	-0.054	-0.059
GLY	-0.094	-0.108	-0.103	-0.094	-0.106	-0.115	-0.135
THR	-0.148	-0.121	-0.102	-0.102	-0.137	-0.134	-0.161
ARG	-0.119	-0.096	-0.084	-0.085	-0.112	-0.158	-0.198
TYR	-0.082	-0.070	-0.065	-0.070	-0.077	-0.075	-0.088
CYS	-0.122	-0.091	-0.090	-0.133	-0.099	-0.101	-0.118
VAL	-0.221	-0.184	-0.14	-0.123	-0.209	-0.198	-0.226
MET	-0.124	-0.101	-0.088	-0.107	-0.112	-0.101	-0.135
TRP	-0.02	-0.021	-0.019	-0.014	-0.009	-0.029	-0.038
PHE	-0.073	-0.068	-0.061	-0.061	-0.067	-0.066	-0.102
ILE	-0.360	-0.313	-0.221	-0.210	-0.313	-0.377	-0.427
LEU	-0.595	-0.501	-0.347	-0.328	-0.524	-0.596	-0.691
LYS	-0.201	-0.164	-0.137	-0.149	-0.19	-0.189	-0.233
PRO	0.120	0.154	0.204	0.237	0.162	0.103	0.078
HYP	0.322	0.244	0.212	0.304	0.254	0.253	0.345
ALA	1.230	0.976	0.773	1.151	1.13	1.232	1.360
GLC	-6.341	-5.374	-18.53	-12.31	-5.825	-4.685	-4.025
LAC	7.480	5.279	10.69	19.08	7.031	4.784	3.432
NH3	1.220	0.717	0.748	0.878	1.072	1.141	1.423
CO2	13.12	11.79	8.920	7.382	11.71	10.83	12.41
RNA	0.062	0.058	0.063	0.057	0.058	0.052	0.062
DNA	0.012	0.012	0.012	0.012	0.012	0.029	0.042
TP	2.421	2.494	2.002	2.243	2.275	2.543	2.769
TC	0.056	0.068	0.053	0.079	0.062	0.079	0.081
LIP	0.151	n.d.	n.d.	n.d.	0.174	0.155	0.139
MAB	0.138	0.112	0.109	0.116	0.115	0.121	0.176

Figure 2a. Viable-cell numbers (closed circles), viability (open diamonds), and dilution rate (---) are shown for 60 days continuous culture. The 2-digit numbers indicate set points for dissolved oxygen (% of air saturation). Samples for metabolite analyses were taken during the last three days of each steady state.

Figure 2b. PMS experiments. Symbols as in legend Figure 2a. PMS was continuously added after day 10. Samples for metabolite analysis were taken at day 14, 15 and 16.

residual value for carbon (C_{rel}) nitrogen (N_{rel}) is given as percentage of the turnover rate.

Carbon balance and carbon-dioxide production rate

In Table 4a it is shown that the carbon balance can be closed for all steady states. In all cases, the relative error for carbon (C_{rel}) is less than 5% of the total carbon turnover. In order to close the C balance, it is critical

to take the bicarbonate in the culture medium into account. If this factor is neglected in the determination of the carbon-dioxide production rate, C_{rel} increases to more than 5% in most cases (Table 4b). Further, the test function h_g (in the determination of which the N balance is also include) increases significantly in all cases (except for the set for the steady state at $pO_2 = 1\%$, which will be discussed below), and to values above 4.61 in 5 out of 6 cases.

Table 2b. Standard deviations of measured metabolic rates of metabolites of entire metabolic network (Values in 10^{-12} mol. cell $^{-1}$.day $^{-1}$)

	pO2 30	pO2 50	pO2 1	pO2 0	pO2 100	pO2 30	PMS
ASP	0.0014	0.0067	0.0034	0.0114	0.0015	0.0176	0.0047
GLU	0.0035	0.0019	0.0069	0.0147	0.0034	0.0160	0.0012
ASN	0.0020	0.0063	0.0079	0.0186	0.0015	0.0597	0.0077
SER	0.0012	0.0035	0.0062	0.0237	0.0031	0.0136	0.0093
GLN	0.0492	0.2145	0.0524	0.2030	0.0779	0.2180	0.1470
HIS	0.0022	0.0060	0.0034	0.0070	0.0021	0.0088	0.0028
GLY	0.0063	0.0110	0.0071	0.0222	0.0049	0.0135	0.0074
THR	0.0024	0.0123	0.0073	0.0192	0.0040	0.0179	0.0095
ARG	0.0011	0.0090	0.0034	0.0202	0.0032	0.0200	0.0119
TYR	0.0007	0.0083	0.0017	0.0115	0.0023	0.0094	0.0060
GYS	0.0078	0.0012	0.0103	0.0061	0.0041	0.0127	0.0075
VAL	0.0043	0.0217	0.0037	0.0213	0.0051	0.0249	0.0102
MET	0.0104	0.0121	0.0033	0.0117	0.0041	0.0116	0.0109
TRP	0.0041	0.0028	0.0017	0.0087	0.0009	0.0004	0.0030
PHE	0.0028	0.0083	0.0033	0.0153	0.0019	0.0073	0.0095
ILE	0.0139	0.0414	0.0053	0.0363	0.0086	0.0431	0.0391
LEU	0.0221	0.0654	0.0094	0.0595	0.0131	0.0688	0.0657
LYS	0.0075	0.0168	0.0025	0.0310	0.0054	0.0188	0.0231
PRO	0.0202	0.0404	0.0246	0.0340	0.0311	0.0069	0.0109
HYP	0.0148	0.0323	0.0098	0.0199	0.0089	0.0404	0.0323
ALA	0.0436	0.1518	0.0233	0.0556	0.0377	0.1780	0.0839
GLC	0.345	0.216	0.435	0.133	0.172	0.284	0.605
LAC	0.640	1.000	0.824	1.610	0.449	0.499	0.208
NH3	0.110	0.142	0.051	0.131	0.121	0.202	0.099
CO2	0.299	1.090	0.167	0.596	0.643	1.220	0.367
RNA	0.006	0.017	0.014	0.025	0.012	0.063	0.004
DNA	0.001	0.001	0.001	0.001	0.001	0.001	0.001
TP	0.019	0.007	0.066	0.102	0.030	0.012	0.071
TC	0.003	0.003	0.002	0.001	0.001	0.001	0.002
LIP	0.016	0.016	0.016	0.016	0.016	0.016	0.014
MAB	0.004	0.005	0.005	0.010	0.001	0.011	0.017

Test for gross measurement errors

For 6 sets of measurement data the test function h_ε , which shows that with more than 90% confidence these data are consistent with respect to both the carbon and the nitrogen balance. Only in one case ($pO_2 = 1\%$, Table 4a) the performance index h_ε is higher than 4.61, indicating that there is a significant measurement error in this set of measured metabolic rates. The relative residual values implies that the measurement of one or more N-containing metabolites are erroneous (Table 4a). The fact that ε_N is positive indicates that either the consumption rate of a (N-containing) metabolite is underestimated or the production rate of a metabolite is overestimated. Comparison of metabolic

rates of N-containing metabolites between the different steady states shown in Table 4a, suggests for example that it is unlikely that the measurement of the total protein (TP) at pO_2 of 1%, is overestimated. It is more probable that either the glutamine-consumption rate is underestimated, or the NH_3 -production rate is overestimated, or a combination of both.

Location and identification of measurement errors

In some cases, gross measurement errors can be located by the serial elimination method (Ripps, 1965; Wang and Stephanopoulos, 1983). With this method it is attempted to locate the source of an error by the recalculation of h_ε for a set of measurement data that has

Table 3a. Average measured metabolic rates of metabolites in underdetermined network. Values (in 10^{-12} mol.cell $^{-1}$.day $^{-1}$) are calculated as described elsewhere (Bonarius et al., 1996). The standard deviations associated with these rates (Table 3b) are linear combinations of the standard deviations of the cellular uptake and production rates, and are calculated accordingly (Box et al., 1978). Abbreviations as in Figure 1

	pO2 30	pO2 50	pO2 1	pO2 0	pO2 100	pO2 30	PMS
G6P	-6.284	-5.302	-7.217	-12.221	-5.758	-4.755	-4.096
G5P	0.628	0.607	0.595	0.554	0.628	0.557	0.651
GAP	0.172	0.172	0.172	0.173	0.172	0.147	0.147
G3P	0.411	0.337	0.210	0.199	0.350	0.346	0.488
PYR	8.078	5.586	0.778	19.541	7.474	5.140	3.965
ACoA	-0.779	-0.588	-0.353	-0.376	-0.673	-0.766	-0.934
CIT	1.467	1.467	1.467	1.467	1.467	1.251	1.251
AKG	-0.650	-0.626	-0.641	-0.947	-0.610	-0.655	-0.633
MAL	0.411	0.423	0.424	0.363	0.423	0.378	0.402
CO2	12.021	10.727	7.484	6.311	10.605	9.523	11.108
ASP	0.139	0.165	0.166	0.204	0.135	0.068	0.207
ASN	0.032	0.026	0.031	0.039	0.040	0.109	0.013
GLU	1.014	0.935	1.000	1.368	0.914	1.026	0.712
GLN	-1.879	-1.391	-1.191	-1.809	-1.696	-1.282	-1.871
NH3	1.209	0.676	0.703	0.848	1.059	1.106	1.397

Table 3b. Standard deviations of metabolic rates of metabolites in underdetermined network (Values in 10^{-12} mol.cell $^{-1}$.day $^{-1}$)

	po2 30	pO2 50	pO2 1	pO2 0	pO2 100	pO2 30	PMS
G6P	0.347	0.218	0.437	0.134	0.172	0.301	0.790
G5P	0.007	0.018	0.018	0.026	0.014	0.063	0.001
GAP	0.035	0.035	0.035	0.035	0.035	0.030	0.030
G3P	0.012	0.017	0.025	0.065	0.014	0.034	0.031
PYR	0.892	1.019	1.053	1.863	0.734	0.954	0.497
ACoA	0.050	0.134	0.033	0.162	0.034	0.142	0.153
CIT	0.147	0.147	0.147	0.147	0.147	0.125	0.125
AKG	0.109	0.309	0.074	0.268	0.124	0.355	0.199
MAL	0.020	0.036	0.024	0.053	0.018	0.083	0.024
CO2	0.514	1.305	0.382	0.811	0.858	1.481	0.628
ASP	0.003	0.008	0.008	0.018	0.004	0.027	0.009
ASN	0.003	0.007	0.010	0.023	0.003	0.061	0.011
GLU	0.153	0.395	0.128	0.371	0.176	0.452	0.265
GLN	0.055	0.225	0.065	0.230	0.083	0.238	0.162
NH3	0.114	0.155	0.063	0.157	0.127	0.222	0.114

been reduced by the removal of data that are suspected to be unsound or erroneous. The removal of the TP, GLN, and NH3 measurement from the data set shown in the third column of Table 4a ($pO_2 = 1\%$) yields a h_e of 1.67, 134, and 0.72, respectively, which indicates that the probability that the ammonia measurement is erroneous is relatively high compared to the likeli-

hood that the total-protein of glutamine determination is incorrect. The differences between these h_e values are however relatively small, and combinations of measurement errors cannot be traced as simultaneous deletion of two measurements is not possible due to the limited number of constraint equations (elemental balances) available.

Table 4. Residual values (estimated errors) of measured carbon (ε_C) and nitrogen (ε_N) metabolized in hybridoma cells. The total carbon (C_{tot}) and nitrogen (N_{tot}) turn-over, the relative residual values (C_{rel} , N_{rel}) and the test function h_ε , are shown as well. In addition, the test function is determined for the same measurements, without taken the bicarbonate buffer into account for the determination of the CO_2 production rates. The erroneous r_{CO_2} (10^{-12} mol.cell $^{-1}$.day $^{-1}$) are given as well

4a. Values for the entire metabolic network.							
	pO ₂ 30	pO ₂ 50	pO ₂ 1	pO ₂ 0	pO ₂ 100	pO ₂ 30	PMS
ε_C	-2.40	-1.99	-6.52	-6.52	-1.60	-0.94	0.35
ε_N	-0.25	0.20	-0.45	-0.39	-0.22	0.50	0.35
C_{tot}	122.40	101.99	125.26	179.87	111.92	99.73	102.97
N_{tot}	14.86	12.28	10.82	12.38	18.30	13.65	16.98
C_{rel}	-2.12	-1.96	-2.47	-3.62	-1.43	-0.94	0.34
N_{rel}	-1.71	1.60	4.12	-3.18	-1.18	3.67	1.20
h_ε	2.96	0.66	15.25	1.87	1.45	0.55	0.32
4b. Values for entire metabolic network, in the case that the bicarbonate buffer in the culture medium has been neglected for the determination of the CO_2 production rate (r_{CO_2})							
r_{CO_2}	21.80	19.68	14.89	13.30	19.45	18.35	20.71
C_{rel}	5.67	5.30	2.18	0.86	5.33	6.16	7.78
h_ε	7.47	2.54	13.96	4.87	13.10	8.44	4.93
4c. Values for the underdetermined subnetwork							
ε_C	2.30	1.78	3.91	-4.11	2.94	2.54	0.98
ε_N	-1.33	-0.96	-0.45	-1.12	-1.20	-0.17	-1.34
C_{tot}	106.11	87.92	110.25	171.60	97.78	82.04	78.91
N_{tot}	6.82	4.61	4.31	6.12	5.58	4.98	6.08
C_{rel}	2.16	2.01	3.55	2.40	3.00	3.10	1.24
N_{rel}	19.53	20.71	10.49	18.28	21.57	2.937	23.00
h_ε	4.58	1.16	1.14	0.82	3.66	0.20	2.30

Van der Heijden and co-workers (1994) recently classified error types in biochemical reaction systems into three categories. When an error is detected by analysis of concentration equations it can either be a measurement error (i), an incorrect system definition (ii), or a result of a too sensitive χ^2 -test due to too small variances (iii). It is not likely that the error in the third set of data (pO₂ = 1%, Table 4a) is an error in system definition. At a probability of at least 90% six different measurement sets (and other data sets (Bonarius et al., 1996, Bonarius et al., 1998a)) do not contain gross errors. This suggests that – at the same probability – the system definition is sound for the metabolites that are relevant for the carbon and nitrogen balance. Another possibility is that the variances of the data set for pO₂ = 1% are too small.

Compared to the other steady states shown in Table 2b, the standard deviation of the NH₃ measurement in the data set for pO₂ = 1% is relatively small. When the standard deviation is increased to $0,202 \cdot 10^{-12}$ mole cell $^{-1}$ day $^{-1}$ (which corresponds to the highest estimated standard deviation for the NH₃ measurement in Table 2b) the test function h_ε decreases from 15,25 to a mere 4,68, which suggests that it cannot be ruled out that the present error in the data for pO₂ = 1% is an error of the third kind. In conclusion, the production and uptake rates of mammalian cells shown here can be tested for the presence of gross errors at a probability of at least 90%, but it is neither possible to locate nor to identify the error(s).

Error analysis of metabolic rates for subnetwork

Table 4c shows the (relative) residual values for carbon and nitrogen and h_e for the smaller, underdetermined network. The set of metabolite flows (Table 3a) calculated from the reaction stoichiometry and the measured production and consumption rate are consistent with respect to the carbon and nitrogen balance. The fact that the test function for each steady state are all below the value of $\chi^2(0.9) = 4.61$ supports that not only the measurements, but also the stoichiometric equations that have been used for the determination of the in- and outgoing flows of the subnetwork are free of gross system errors (at a probability of at least 90%), and can be used for flux analysis.

It is further noteworthy that in contrast to the ε_N values corresponding to the entire network (Table 4a), *all* ε_N values associated with the underdetermined subnetwork (Table 4c) are negative. In other words, according to the N balance, some of the N-producing fluxes in the other reactions in cell metabolism than those of the subnetwork, are overestimated (or N-consuming reactions are underestimated). Because this seems not to be the case for C-containing metabolites, a possible explanation for this small though systematic difference is either that a reaction in which NH_3 is consumed has incorrectly been neglected, or that a NH_3 -producing pathway has incorrectly been added to the network. Examples of the latter could be the degradation pathways of amino acids such as cysteine and threonine (Figure 1), which both yield NH_3 .

Conclusion

The carbon balance can be closed in continuous mammalian-cell culture, provided that the bicarbonate buffer in the culture medium is taken into account for the determination of the CO_2 production rate. Together with the nitrogen balance, this allows to test the measurement data of metabolites that are relevant for flux analysis on the presence of gross errors.

The carbon and nitrogen balances over the metabolic subnetwork that consists of the glycolysis, the TCA cycle, the pentose phosphate pathway, and the glutaminolysis, can be closed within the 90% confidence interval.

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