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ORIGINAL PAPER

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^{13}C NMR studies of the fluxes in the central metabolism of *Corynebacterium glutamicum* during growth and overproduction of amino acids in batch cultures

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Abstract The carbon flux distribution in the central metabolism of *Corynebacterium glutamicum* was studied in batch cultures using [1-^{13}C]- and [6-^{13}C]glucose as substrate during exponential growth as well as during overproduction of L-lysine and L-glutamate. Using the ^{13}C NMR data in conjunction with stoichiometric metabolite balances, molar fluxes were quantified and normalised to the glucose uptake rate, which was set to 100. The normalised molar flux via the hexose monophosphate pathway was 40 during exponential growth, whereas it was only 17 during L-glutamate production. During L-lysine production, the normalised hexose monophosphate pathway flux was elevated to 47. Thus, the carbon flux via this pathway correlated with the NADPH demand for bacterial growth and L-lysine overproduction. The normalised molar flux in the tricarboxylic acid cycle at the level of 2-oxoglutarate dehydrogenase was 100 during exponential growth and 103 during L-lysine secretion. During L-glutamate formation, the normalised flux through the tricarboxylic acid cycle was reduced to 60. In contrast to earlier NMR studies with *C. glutamicum*, no significant activity of the glyoxylate pathway could be detected. All experiments indicated a strong in vivo flux from oxaloacetate back to phospho*enol*pyruvate and/or pyruvate, which might be due to phospho*enol*-pyruvate carboxykinase activity in *C. glutamicum*.

Introduction

Corynebacterium glutamicum and its close relatives *C. glutamicum* ssp. *flavum* and *lactoferm.*, are widely used for the industrial production of the amino acids L-lysine and L-glutamate. The key reactions of the central metabolism and their regulation have been intensively studied in coryneform bacteria. These organisms were shown to possess the Embden-Meyerhof glycolytic pathway, the hexose monophosphate pathway (HMP), the tricarboxylic acid (TCA) cycle and the glyoxylate bypass (Kinoshita 1985). The TCA cycle requires a continuous replenishment of oxaloacetate in order to replace the intermediates withdrawn for the synthesis of biomass and amino acids. This anaplerotic function is fullfilled by phospho*enol*pyruvate carboxylase (Ozaki and Shiio 1969) and a pyruvate-carboxylating enzyme (Tosaka et al. 1979). In spite of this detailed biochemical knowledge, the study of in vivo metabolite fluxes in *C. glutamicum* has thus far received only relatively little interest. Studies using NMR (Walker et al. 1982; Inbar et al. 1985; Yamaguchi et al. 1986; Inbar and Lapidot 1987; Ishino et al. 1991) used various strains of *C. glutamicum* and closely related organisms, and the reported fluxes through the HMP and TCA cycles and the glyoxylate pathway differ typically by more than 50%. Using an alternative approach, based on stoichiometric modelling techniques, results were obtained for *C. glutamicum* during L-lysine overproduction that agree with the NMR studies concerning HMP activity but indicate twice as high a TCA cycle flux (Vallino and Stephanopoulos 1990, 1993). In summary, no conclusive data about the intracellular fluxes in *C. glutamicum* during amino acid overproduction as compared to exponential growth are yet available.

Therefore, the purpose of this study was to determine the intracellular fluxes in the central metabolism of isogenic strains of *C. glutamicum* in batch cultures during exponential growth as well as during amino acid overproduction in a single comprehensive study, using an analysis by combined NMR and metabolite-balancing methods that, in contrast to the studies mentioned above, is independent of biochemical constraints and

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uses an exact mathematical description of the ^{13}C isotope labelling.

Materials and methods

Bacterial strains and culture conditions

C. glutamicum ATCC 13032 (wild-type) was used for growth studies and L-glutamate production. *C. glutamicum* MH20-22B, derived from ATCC 13032 by undirected mutagenesis (Schrumpf et al. 1992), was used as an L-lysine-overproducing strain (Schrumpf et al. 1991). The minimal media used were as described previously for exponential growth (Eikmanns et al. 1991), for L-glutamate production (Gutmann et al. 1992) and for L-lysine production (Schrumpf et al. 1992). In these media, the carbon source was replaced by [1-^{13}C]glucose or [6-^{13}C]glucose (99% enriched, Omicron, USA) at a final concentration of 20 g/l. C. glutamicum cells were grown aerobically as 60-ml cultures in 500-ml baffled conical flasks at 30°C on a rotary shaker at 140 rpm. The absorbance was measured at 600 nm to determine the bacterial growth. Glucose consumption and amino acid formation were determined enzymatically. The cells were harvested in the exponential growth phase or during production of L-glutamate or L-lysine. Cytosolic glutamate and alanine were extracted from cells by perchloric acid treatment and isolated by preparative fast performance liquid chromatography for NMR analysis (Sonntag et al. 1993).

NMR spectroscopy

All NMR spectra were run using a Bruker AMX-400 WB spectrometer equipped with a 5-mm ^{1}H inverse probe head. ^{13}C enrichments of the protonated carbons of glutamate and alanine were determined from normal and ^{13}C-decoupled 400-MHz ^{1}H spectra as described previously (Sonntag et al. 1993). A relaxation delay of 30 s was used to avoid overpulsing of the centre-band proton signals (i.e. those bonded to ^{12}C) (Walker et al. 1982).

Determination of metabolite fluxes

From the measured NMR data, biomass accumulation, glucose consumption and amino acid production the fluxes in the central metabolism were determined separately for each physiological state as described below using the metabolic model schematically shown in Fig. 1A.

All fluxes were expressed in molar units and normalised to the measured glucose consumption rate, which was set to 100. The fluxes of precursors withdrawn from the central metabolism for biomass synthesis were calculated from the measured biomass yield using the stoichiometric approach of Neidhardt (Neidhardt et al. 1982). The fluxes of L-lysine and L-glutamate synthesis were set equal to the measured accumulation of these amino acids in the medium. To determine the flux through the HMP, two complementary experiments using [1-^{13}C]glucose and [6-^{13}C]glucose were performed (Yamaguchi et al. 1986) for each different physiological state. Following the approach of Walker (Walker et al. 1982), the fraction of glucose metabolised over HMP, relative to the fraction metabolised in glycolysis, was calculated as $(1 - A/B)$, where A and B represent the ^{13}C labelling in C-3 of pyruvate obtained from the experiments with [1-^{13}C]glucose and [6-^{13}C]glucose respectively. Since pyruvate could not be isolated from cell extracts, the ^{13}C labelling of C-4 of glutamate and/or C-3 of alanine, which are both directly derived from C-3 of pyruvate, were determined.

The remaining unknown fluxes, i.e. via the TCA cycle, the glyoxylate pathway and the anaplerotic reactions, were determined by a method that combines ^{13}C NMR spectroscopy and metabolite balancing. The procedure is described in a preliminary form in Marx et al. (1993, 1994). A paper on metabolic flux analysis of microorganisms in continuous culture that also describes the complete mathematical details of our procedure is submitted for publication elsewhere (Marx et al. 1995). Whereas the latter two publications (Marx et al. 1994, 1995) cover a single, well-defined physiological state of *C. glutamicum* in continuous culture and in part concentrate on the methodology of flux determination, the present paper describes and enables the direct comparison of three different physiological states under batch-fermentation conditions. The flux determination method is based on the fact that the percentage ^{13}C atom enrichments in the intermediates of the central metabolism can be accurately calculated for each possible flux configuration in the metabolic network provided that a metabolic and isotopic steady state is present (Marx et al. 1993, 1994). A corresponding ^{13}C label calculation algorithm was coupled with an iterative parameter-optimisation algorithm to determine the flux distribution in the complete metabolic network that yielded an optimal fit of the calculated ^{13}C atom enrichments to the experimental NMR data. The metabolic model shown in Fig. 1A was used in the present study. The error function that was minimised by the non-linear least-squares algorithm consisted of the sum of the squared differences between corresponding calculated and experimental data (^{13}C data as well as measured fluxes). These differences were normalised to the respective experimental errors. It is important to point out that the experimental data that were taken into account for parameter optimisation included not only the ^{13}C NMR data of glutamate and alanine but also the measured fluxes of glucose consumption, of product accumulation and of biomass generation as well as the value calculated for the HMP flux. For all flux distributions encountered during the optimisation procedure, the algorithm automatically performed metabolite balancing at each branch point of the metabolic network. The parameter optimisation, as described, yielded optimised values for all fluxes in the network, i.e. also for the measured fluxes. The complete mathematical details of our analysis are available on request.

Special features of the ^{13}C label modelling

Apart from the pathways mentioned in the Introduction and indicated in Fig. 1A, the following features of our metabolic model were essential elements in the analysis because of their influence on the ^{13}C labelling in the network:

1. Both phospho*enol*pyruvate (PEP) and pyruvate were alternative substrates for anaplerotic carboxylating reactions leading to oxaloacetate, because it has been shown that both PEP carboxylase and a pyruvate-carboxylating enzyme are present in *C. glutamicum* (Ozaki and Shiio 1969; Tosaka et al. 1979; Peters-Wendisch et al. 1993).

2. A possible back flux of oxaloacetate to PEP or pyruvate was included as a parameter in the model, because the gluconeogenetic enzyme PEP carboxykinase (Jetten and Sinskey 1993; Peters-Wendisch et al. 1993) and oxaloacetate decarboxylase (Jetten et al. 1994) were found to be present with relatively high activity in *C. glutamicum.*

3. An exchange in the malate dehydrogenase and fumarase reactions was allowed for in the model because its presence is likely from literature data (Walsh and Koshland 1984).

These features have not been previously considered in NMR studies of fluxes in *C. glutamicum.*

Results

Flux analysis during exponential growth

During exponential growth, the specific glucose consumption rate was 3.8 ± 0.4 mmole . g dry weight^{-1} . h^{-1}

Table 1 ^{13}C enrichments in carbons C-2, C-3 and C-4 of intracellular glutamate as well as in carbons C-2 and C-3 of intracellular alanine obtained after incubation of *C. glutamicum* with [1-^{13}C]- or [6-^{13}C]glucose as indicated during exponential growth, L-glutamate production and L-lysine production. *ND* not determined

Glucose	Glutamate ^{13}C enrichments[a] (%)			Alanine ^{13}C enrichments (%)	
	C-2	C-3	C-4	C-2	C-3
Exponential growth					
[1-^{13}C]	24.3	12.0	30.6	ND	ND
[6-^{13}C]	38.7	19.8	51.2	ND	ND
L-Glutamate production					
[1-^{13}C]	35.9	10.1	42.3	2.7	43.4
[6-^{13}C]	43.8	13.2	51.1	2.5	51.4
L-Lysine production					
[1-^{13}C]	23.6	10.5	24.4	3.7	27.4
[6-^{13}C]	ND	ND	ND	ND	50.6

[a] The absolute experimental inaccuracy was estimated to be 0.8% for all label determinations.

and the biomass yield was determined to be 0.37 ± 0.03 g dry weight/g glucose. Since by-products were not accumulated, the glucose was converted solely to biomass and CO_2. From these data, the normalised fluxes of precursors for anabolism were calculated. To quantify the specific ^{13}C enrichments in the precursor metabolite 2-oxoglutarate, intracellular glutamate was isolated from perchloric acid extracts of washed cells and subjected to NMR analysis. The ^{13}C atom enrichments in cytosolic glutamate thus obtained from cells harvested at an A_{600} of 10 (2.9 mg dry weight/ml), are shown in Table 1. From the enrichments in glutamate C-4 found after incubation with [6-^{13}C]glucose and [1-^{13}C]glucose, it was calculated that at the glucose 6-phosphate branch point, $39 \pm 2\%$ of the glucose was metabolised over HMP and $61 \pm 2\%$ over glycolysis.

The values for the anabolic fluxes as well as the HMP partition, together with the ^{13}C labelling data, were supplied as measurement data to the parameter optimisation program to determine finally the complete flux distribution that optimally corresponded with the measurement data. The result is shown in Fig. 1A. To avoid ambiguity, the flux through 2-oxoglutarate dehydrogenase is henceforth referred to as TCA cycle flux. Thus, the parameter optimisation algorithm determined a normalised molar flux through the hexose monophosphate pathway of 40, the glucose consumption rate being normalised to 100. The normalised molar flux through glycolysis, expressed in terms of C_3 carbon units, was 170. This is equivalent to a normalised flux of 85 in C_6 units, indicating that, at the triose phosphate level, 15% of the total carbon supplied had already been drawn off from the central metabolism for biomass synthesis and CO_2 production.

The normalised molar flux in the tricarboxylic acid cycle was 100, that of 2-oxoglutarate used for biosynthetic purposes was 8. The high TCA cycle flux explains the significant ^{13}C enrichment of glutamate C-3 (Table 1), which (in the absence of glyoxylate pathway activity) would have been close to zero if no TCA cycle activity had been present.

The pools of pyruvate and PEP could be combined without influencing the results of the parameter optimisation. Therefore, the anaplerotic fluxes involving these pools were added together. The results of the parameter optimisation indicated a total normalised anaplerotic flux of 20. Unexpectedly the analysis showed that a high fraction of 46% of the total efflux from the oxaloacetate pool was directed to the PEP/pyruvate pool via decarboxylating reactions. From this, it was calculated that the normalised exchange flux between oxaloacetate and PEP/pyruvate was at least 125 (Fig. 1A). Since the normalised net anaplerotic flux was 20, the flux of PEP/pyruvate to oxaloacetate was therefore at least 145. This is consistent with the fact that C-2 of glutamate was only slightly less enriched than C-4 (Table 1). The C-2 label would have been much lower if the total flux of PEP/pyruvate to oxaloacetate had been only 20, i.e. the net anaplerotic flux. The flux through isocitrate lyase and malate synthase was found to be practically 0, indicating that the glyoxylate pathway was not operative as an anaplerotic sequence under our conditions. The flux of oxaloacetate to fumarate, with associated label scrambling, amounted to 10% of the total efflux from the oxaloacetate pool. From this it was calculated that the normalised flux via back-reactions of malate dehydrogenase and fumarase was at least 25 (Fig. 1A). The goodness of fit between experimental and calculated data can be judged from the average residual of the fit for the ^{13}C label data. Ideally, a value of less than 1 would indicate that all calculated values lie within the error bounds of the experimental data. For the experiment with [1-^{13}C]glucose, a fairly good residual of 1.8 was obtained. Although the residual for the [6-^{13}C]glucose experiment was higher (3.0), the estimated fluxes for both experiments were identical, showing the consistency of the data. The validity of the flux model is further supported by the fact that inversion of irreversible reactions (e.g. malate synthase, citrate synthase, pyruvate dehydrogenase) did not occur, although the model in principle allowed for it.

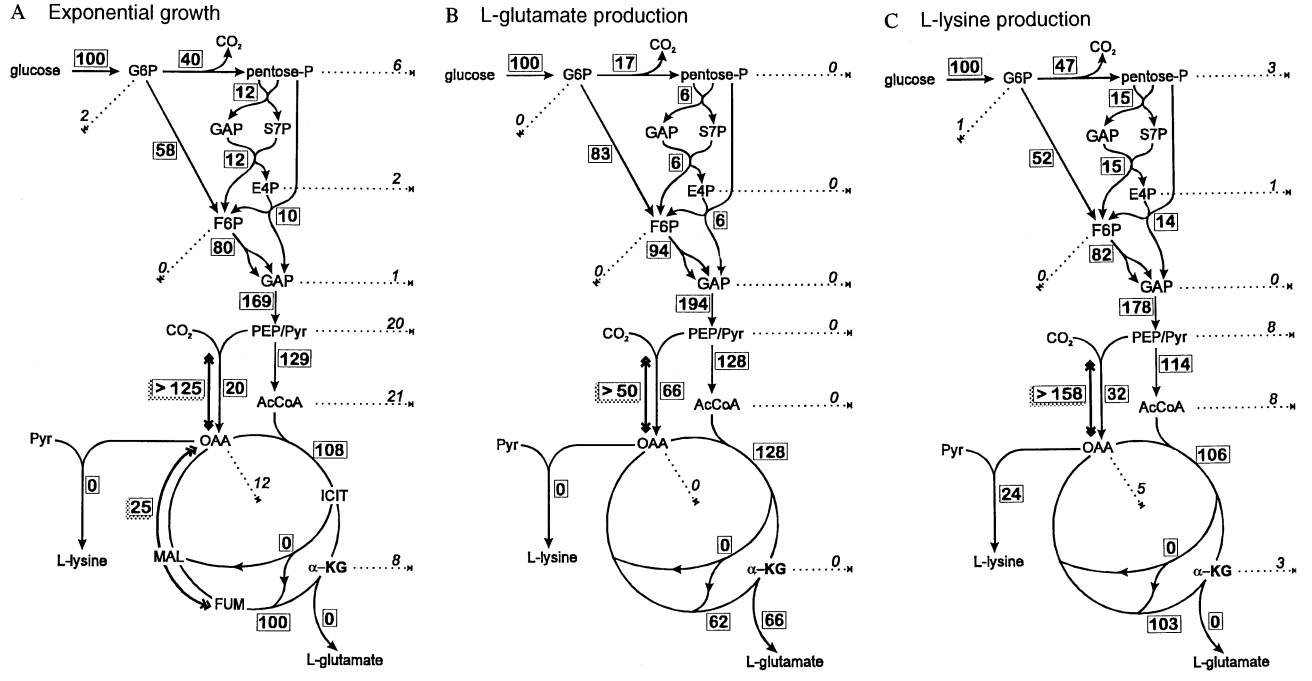

Fig. 1 **A** Schematic representation of the central metabolism of *C. glutamicum* used for flux determinations in this study. The numbers represent the flux distribution determined during exponential growth in *C. glutamicum* ATCC 13032 (wild-type). **B** Flux distribution determined during L-glutamate production in *C. glutamicum* ATCC 13032 under biotin limitation. **C** Flux distribution determined during lysine production in *C. glutamicum* MH20-22B. All fluxes are on a molar basis and were normalised to the glucose consumption rate. *Dotted arrows* fluxes of precursors withdrawn for biomass synthesis. *Double-headed arrows with numbers in shaded boxes* exchange fluxes that are superimposed on the corresponding net fluxes. *G6P* glucose 6-phosphate, *GAP* glyceraldehyde 3-phosphate, *S7P* sedoheptulose 7-phosphate, *E4P* erythrose 4-phosphate, *F6P* fructose 6-phosphate, *PEP* phospho*enol*pyruvate, *Pyr* pyruvate, *AcCoA* acetyl-coenzyme A, *OAA* oxaloacetate, *α-KG*: 2-oxoglutarate, *ICIT* isocitrate, *MAL* malate, *FUM* fumarate

To investigate whether the flux distribution changed during the exponential growth phase, the experiments were also performed with cells harvested at two different cell densities, i.e. at $A_{600} = 5$ and at $A_{600} = 20$. For all four experiments the data analysis yielded a flux distribution that was practically identical to that found with cells harvested at $A_{600} = 10$.

Flux analysis during L-glutamate production

To determine the flux distribution in the wild-type strain of *C. glutamicum* during L-glutamate production, experiments with [1-^{13}C]- and [6-^{13}C]glucose were performed with cells under biotin limitation (Shiio et al. 1962). The glucose consumption rate was 1.8 ± 0.2 mmol . g dry weight^{-1} . h^{-1} and the molar yield of L-glutamate on glucose was $60 \pm 10\%$. Since no cell growth occurred, the precursor requirements for biomass synthesis did not have to be considered in the flux determination. To increase the reliability of the analysis, the ^{13}C enrichments were not only determined from intracellular glutamate but also from intracellular alanine. The percentage atom enrichments, as determined by NMR, are given in Table 1. Comparing the [1-^{13}C]glucose data to those for exponential growth shows that the total ^{13}C enrichment in glutamate was much higher during L-glutamate production, indicating that less ^{13}C label was lost as CO_2 due to HMP activity. A correspondingly lower normalised flux through the HMP of only 16 ± 2 was calculated from the [1-^{13}C]- and [6-^{13}C]glucose data.

The values for L-glutamate production and HMP flux, together with the ^{13}C atom enrichments, were used for the parameter-optimisation program. A good fit to the data was obtained (the average residual for the ^{13}C labellings was only 1.1–1.6) with almost identical flux estimations for the two experiments, indicating that the data were highly consistent. The resulting flux distribution is shown in Fig. 1B. As optimal values for the normalised measured fluxes, the parameter-optimisation program found 17 for the HMP and 66 for the formation of L-glutamate.

The significantly lower labelling of glutamate C-3, determined in the experiment with [6-^{13}C]glucose (Table 1), as compared to the experiment during exponential growth is indicative of a reduced TCA cycle flux during L-glutamate production. Consistent with this, the parameter-optimisation program yielded a normalised TCA cycle flux of 62, i.e. about 40% lower than during exponential growth. No oxaloacetate label scrambling due to exchange reactions with fumarate

was present, indicating that the flux of oxaloacetate via the reverse malate dehydrogenase and fumarase reactions was negligible.

The value obtained for the normalised net anaplerotic flux was 66, which is equal to the L-glutamate production because no TCA cycle intermediates were withdrawn for biomass formation. A significant percentage (29%) of the total efflux from the oxaloacetate pool was back to PEP/pyruvate. From this, it was calculated that the normalised exchange flux between oxaloacetate and PEP and pyruvate was at least 50 (Fig. 1B). As during exponential growth, no significant flux through the glyoxylate pathway was found.

Flux analysis during L-lysine production

The mutant strain MH20-22B of *C. glutamicum* was used for flux analysis under L-lysine production conditions. The glucose consumption was 1.1 ± 0.2 mmol. g dry weight^{-1}.h^{-1} and the L-lysine molar yield on glucose was $25 \pm 5\%$. During L-lysine production, some growth still took place, with a cell yield of 0.15 ± 0.05 g biomass/g glucose. From the cell yield, the fluxes of precursor metabolites for anabolism were calculated. Besides L-lysine, no by-products accumulated in significant amounts, showing that glucose was converted mainly to biomass, L-lysine and CO_2. To determine the HMP flux, the ^{13}C enrichments in intracellular glutamate and alanine after feeding [1-^{13}C]- and [6-^{13}C]glucose were determined (Table 1). The overall labelling of L-glutamate synthesised from [1-^{13}C]glucose during L-lysine production was somewhat lower than during exponential growth. From the ^{13}C data, an elevated normalised flux through the HMP of 46 ± 2 was calculated.

These measured flux data and ^{13}C atom enrichments were used in the parameter-fitting procedure to yield the flux distribution shown in Fig. 1C. The average residual of the fit was 1.8. Compared to the flux distribution during exponential growth in the wild-type strain, an 18% higher HMP flux was found (47 compared to 40) whereas the normalised TCA flux was comparable (103). As under conditions of L-glutamate production, the flux of oxaloacetate via the reversed malate dehydrogenase and fumarase reactions was negligible, thus no label scrambling in oxaloacetate due to exchange with fumarate was present. The flux through the glyoxylate pathway was insignificant.

Owing to the greatly increased amounts of oxaloacetate required for L-lysine production, the normalised anaplerotic flux was 1.5 times higher than during exponential growth, i.e. 32 compared to 20. Of the total efflux from the oxaloacetate pool, 54% was to PEP/pyruvate, from which it was calculated that the normalised exchange flux between those pools was at least 158 (Fig. 1C). This was slightly higher than the value of 125 found during exponential growth.

Discussion

It has been previously observed (Ishino et al. 1991) that the relative flux distribution between the HMP and glycolysis correlates with the respective NADPH requirements for L-lysine production and L-glutamate production. This requirement is expected to be low during L-glutamate production and high during growth, and especially under L-lysine production conditions. According to the bacterial cell composition (Neidhardt et al. 1982), 18.2 mmol NADPH is needed for the synthesis of 1 g cell dry weight; 4 mol NADPH is required to synthesise 1 mol lysine from pyruvate and oxaloacetate; 1 mol NADPH is required to synthesise 1 mol of glutamate from 2-oxoglutarate. On the other hand, 1 mol NADPH is produced/mol of isocitrate oxidised by the NADP-dependent isocitrate dehydrogenase in *C. glutamicum*, and 2 mol of NADPH are produced/mol glucose metabolised in the oxidative HMP pathway. The total amounts of NADPH that were required and produced per mole of glucose are shown in Table 2. Thus, from our flux data it is apparent that the NADPH synthesis considerably exceeded the demand for growth as well as for metabolite overproduction in batch cultures. It is not understood how the organism solves this imbalance problem. To our knowledge, dinucleotide transhydrogenases have not been detected so far in *C. glutamicum*, nor has NADPH oxidase activity been observed in vitro. The possibility remains that membrane-bound NADPH oxidase activity, not observable in extracts, is present in vivo. Therefore, according to our results stoichiometric balancing of NADPH probably cannot be used as a constraint in the determination of flux distributions by metabolic balancing.

The normalised molar flux through the HMP of 40, found during exponential growth in *Corynebacterium glutamicum* (ATCC 13032), agrees well with the value of 45 found in a recent study with *Corynebacterium melassecola* (Rollin et al. 1995). The normalised HMP fluxes found during L-glutamate and L-lysine production are also in accordance with literature data for *M. ammoniaphilum* (Walker et al. 1982) and *C. glutamicum* (Yamaguchi et al. 1986).

Table 2 Total amounts of NADPH (mol/mol of glucose consumed) required and produced according to the flux distributions determined in this study (Fig. 1A–C)

Condition	NADPH (mol/mol)		
	Required	Produced	Excess
Exponential growth	1.21	1.88	0.67
L-Glutamate production	0.66	1.61	0.95
L-Lysine production	1.45	2.00	0.55

During exponential growth, the molar flux, normalised to the glucose consumption rate, through the TCA cycle (i.e. at the level of 2-oxoglutarate dehydrogenase) was 100. During L-glutamate production, the normalised TCA cycle flux was 62, i.e. 40% less than during exponential growth. This result corresponds with the suggestion (Walker et al. 1982; Kinoshita 1985) that L-glutamate accumulation is partly due to a reduced activity of 2-oxoglutarate dehydrogenase. Since, during L-glutamate production, the normalised anaplerotic flux was 66 and the normalised TCA cycle flux 62 (Fig. 1B), it follows that the relative contribution of the TCA cycle to the ^{13}C labelling of the finally synthesised glutamate was 48%. This differs from the 16% found in the analysis of *Microbacterium ammoniaphilum* (Walker et al. 1982), probably because of the difference in methods used for ^{13}C label analysis. The normalised TCA cycle flux of 103 found during L-lysine production is 30% lower than described previously for comparable physiological conditions (Vallino and Stephanopoulos 1993). However, the latter study was exclusively based on metabolite balances and, as a biochemical constraint, assumed that NADPH synthesis and consumption were precisely matched, which according to our results may not apply in vivo.

Several studies reported high glyoxylate cycle activity during growth of *C. glutamicum* on glucose. The glyoxylate cycle was found to contribute 26% to the L-glutamate synthesised (Walker et al. 1982) by *M. ammoniaphilum*. Data obtained by Yamaguchi et al. 1986) suggested that about 50% of the flux through citrate synthase was diverted through the glyoxylate bypass during L-lysine production with *C. glutamicum*. However, the analysis of the glutamate ^{13}C atom enrichments found in the present study clearly indicated that, in all experiments performed, no significant activity of the glyoxylate cycle in vivo was present. This result is in accordance with the fact that the specific activities of the glyoxylate pathway enzymes are more than 50-fold reduced during growth on glucose as compared to acetate as the sole carbon source (Reinscheid et al. 1994a, b). Similar results were obtained in a very recent study of exponentially growing *C. melassecola* (Rollin et al. 1995).

The net anaplerotic fluxes reported in this study are equal to the total requirement of TCA cycle intermediates as precursors for the synthesis of biomass and amino acids. These fluxes were accurately determined by the metabolite balancing that was an integral part of our analysis. The presence of the flux from oxaloacetate back to PEP/pyruvate in *C. glutamicum* has not been reported before and was also found in our study of *C. glutamicum* in continuous culture (Marx et al. 1995). To assess whether it can unequivocally be concluded from our experimental data that this flux existed in vivo, parameter optimisations were carried out using a metabolic model that did not include the flux from oxaloacetate back to PEP/pyruvate. In this case, it proved not to be possible to fit the model to the data, confirming that a significant back flux of oxaloacetate to the PEP/pyruvate pool must have been present. Walker (Walker et al. 1982) observed ^{13}C labellings in C-4 and C-5 of glutamate synthesised by *M. ammoniaphilum* that could also be explained by a recycling of oxaloacetate to PEP/pyruvate. The conversion of oxaloacetate to PEP was also proposed in a recent study of oxaloacetate-converting enzyme activities in *C. glutamicum* (Jetten et al. 1994), where the enzyme PEP carboxykinase was found to be present at high activity in *C. glutamicum*. However, whereas those studies did not allow any conclusions to be drawn about in vivo fluxes, the present study provides strong, quantitative evidence of the existence of a flux from oxaloacetate back to PEP/pyruvate in vivo in *C. glutamicum* under glycolytic conditions in batch cultures. This flux was present during exponential growth as well as during L-glutamate and L-lysine production. The high flux values found indicate that a rapid interconversion of oxaloacetate, phospho*enol*pyruvate and pyruvate was present.

References

Eikmanns BJ, Metzger M, Reinscheid D, Kircher M, Sahm H (1991) Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. Appl Microbiol Biotechnol 34:617–622

Gutmann M, Hoischen C, Krämer R (1992) Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim Biophys Acta 1112:115–123

Inbar L, Lapidot A (1987) ^{13}C-NMR, ^{1}H-NMR and gas-chromatography mass-spectrometry studies of the biosynthesis of ^{13}C-enriched L-lysine by *Brevibacterium flavum*. Eur J Biochem 162:621–633

Inbar L, Kahana Z, Lapidot A (1985) Natural abundance ^{13}C-nuclear magnetic resonance studies of regulation and overproduction of L-lysine by *Brevibacterium flavum*. J Biochem (Tokyo) 149:601–607

Ishino S, Shimomura-Nishimuta J, Yamaguchi K, Araki K (1991) ^{13}C-NMR studies of glucose metabolism in L-glutamic acid and L-lysine fermentation by *Corynebacterium glutamicum*. J Gen Appl Microbiol 37:157–165

Jetten MSM, Sinskey AJ (1993) Characterisation of phospho*enol*-pyruvate carboxykinase from *Corynebacterium glutamicum*. FEMS Microbiol Lett 111:183–188

Jetten MSM, Pitoc GA, Follettie MT, Sinskey AJ (1994) Regulation of phospho(*enol*)-pyruvate- and oxaloacetate-converting enzymes in Corynebacterium glutamicum. Appl Microbiol Biotechnol 41:47–52

Kinoshita S (1985) Glutamic acid bacteria. In: Demain AL, Solomon NA (eds) Biology of industrial microorganisms. Benjamin Cummings, London, pp 115–142

Marx A, Sonntag K, Graaf AA de, Wiechert W, Sahm H (1993) Simulation of ^{13}C labelling for NMR studies of the pentose phosphate pathway. Biol Chem Hoppe-Seyler 374:689

Marx A, Graaf AA de, Wiechert, W Eggeling, L Sahm H (1994) Carbon flux in the lysine producing strain *Corynebacterium glutamicum* MH20-22B: analysis by ^{13}C NMR of amino acids and metabolite balancing. In: Gnaiger E, Gellerich FN, Wysss M (eds) What is controlling life?. Modern trends in bio thermokinetics vol 3. Innsbruck University Press, Innsbruck, Austria, pp 240–243

Marx A, Graaf AA de, Wiechert, W Eggeling, L Sahm H (1995) Determination of the fluxes in the central metabolism of *Corynebacterium glutamicum* by NMR spectroscopy combined with metabolite balancing. Biotechnol Bioeng

Neidhardt FC, Ingraham JL, Schächter M (1982) Physiology of the bacterial cell. Sinauer, Sunderland, Massachusetts

Ozaki H, Shiio I (1969) Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum*. J Biochem (Tokyo) 66:297–311

Peters-Wendisch PG, Eikmanns BJ, Thierbach G, Bachmann B, Sahm H (1993) Phospho*enol*pyruvate carboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production. FEMS Microbiol Lett 112:269–274

Reinscheid D, Eikmanns BJ, Sahm H (1994a) Characterisation of the isocitrate lyase gene from *Corynebacterium glutamicum* and biochemical analysis of the enzyme. J Bacteriol 176:3474–3483

Reinscheid D, Eikmanns BJ, Sahm H (1994b) Malate synthase from *Corynebacterium glutamicum*: sequence analysis of the gene and biochemical characterization of the enzyme. Microbiologia 140:3099–3108

Rollin C, Morgant V, Guyonvarch A, Guerquin-Kern J-L (1995) ^{13}C-NMR studies of *Corynebacterium melassecola* metabolic pathways. Eur J Biochem 227:488–493

Schrumpf B, Schwarzer A, Kalinowski J, Pühler A, Eggeling L, Sahm H (1991) A functionally split pathway for lysine synthesis in *Corynebacterium glutamicum*. J Bacteriol 173:4510–4516

Schrumpf B, Eggeling L, Sahm H (1992) Isolation and prominent characteristics of an L-lysine hyperproducing strain of *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 37:566–571

Shiio I, Otsuka SI, Katsuya M (1962) Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and the cellular permeability of amino acids. J Biochem (Tokyo) 51:56–62

Sonntag K, Eggeling L, Graaf AA de, Sahm H (1993) Flux partitioning in the split pathway of lysine synthesis in *Corynebacterium glutamicum*, quantification by ^{13}C and ^{1}H-NMR spectroscopy. Eur J Biochem 213:1325–1331

Tosaka O, Morioka H, Kakinami K (1979) The role of biotin-dependent pyruvate carboxylase in L-lysine production. Agric Biol Chem 43:1513–1519

Vallino JJ, Stephanopoulos G (1990) Flux determination in cellular bioreaction networks: application to lysine fermentation. In: Sikdar SK, Bier M, Todd P (eds) Frontiers in bioprocessing, CRC Press, Boca Raton, Fla, pp 205–219

Vallino JJ, Stephanopoulos G (1993) Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. Biotechnol Bioeng 41:633–646

Walker TE, Han CH, Kollman VH, London RE, Matwiyoff NA (1982) ^{13}C NMR studies of the biosynthesis by *Microbacterium ammoniaphilum* of L-glutamate selectively enriched with carbon-13. J Biol Chem 257:1189–1195

Walsh K, Koshland DE Jr (1984) Determination of flux through the branch point of two metabolic cycles. J Biol Chem 259:9646–9654

Yamaguchi K, Ishino S, Araki K, Shirahata K (1986) ^{13}C-NMR studies of lysine fermentation with a *Corynebacterium glutamicum* mutant. Agric Biol Chem 50:2453–2459