Flux analysis of underdetermined metabolic networks: the quest for the missing constraints

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Traditionally, the intracellular fluxes of complex metabolic networks were quantified by isotopic-tracer experiments, but, owing to practical limitations, 'metabolic-flux balancing' is emerging as an alternative. This has become an important tool for the quantitative analysis of the physiology of microorganisms and mammalian cells. It has been successfully applied to finding potential sites for metabolic engineering, determining metabolic capabilities and designing optimal feeding strategies. However, it has the fundamental problem that metabolic networks, and cyclic metabolic pathways in particular, are underdetermined. The search for constraints that can be used to determine fluxes correctly for a range of different conditions is an exciting challenge.

Microorganisms are widely exploited for the synthesis of proteins or low molecular weight compounds and to transform substrates into a wide variety of products. As with chemical-production plants, the flux distribution determines the efficiency of the production process. In biological systems, nutrients are only partly used for biomass and product synthesis, the remainder being used for energy supply. In addition, waste products accumulate and significant energy dissipates in futile cycles. In order to optimize the capacity of microbial metabolism, and thus increase yields, the intracellular metabolite flows have to be quantitated.

Traditionally, metabolic fluxes have been determined by isotopic-tracer experiments. As the carbon stoichiometry of metabolic reactions is known, it is possible to determine the fluxes at certain branch points by tracing the metabolic fate of carbon-labelled substrates. Although these isotopic-tracer techniques are well established and have been significantly improved, particularly by the application of nuclear magnetic resonance (NMR) technology to biological systems[1], they are laborious and expensive to conduct and cannot be used on an industrial scale. As an alternative, 'metabolic-flux balancing'[2], which requires the measurement of only the extracellular metabolites (Box 1), has been proposed as a means of determining the flow through the primary metabolic pathways[3].

Data from isotopic-tracer experiments have been supplemented with mass-balance equations of the relevant metabolites for the determination of fluxes in entire metabolic networks[4–7]. Additionally, computational methods have been developed to combine these different types of information, allowing the calculation of metabolic fluxes with a minimum of algebraic manipulation[5,6]. However, for industrial applications, it is desirable to determine intracellular metabolic fluxes by metabolic-flux balancing alone, and, because no isotopically labelled substrate is required, the estimation of fluxes from mass balances (see Glossary) can be made in virtually any biological or reactor system regardless of scale.

On-line applications of 'crude' stoichiometric analysis have already been shown to be effective as a means of improving production yields in fermentation processes[8]. It is theoretically possible to measure and control metabolic conversions on the level of single enzymatic reactions by metabolic-flux-balancing techniques, with the measurement of only a limited number of extracellular metabolites[9]. Apart from controlling complex metabolic networks or tracing potential sites for metabolic engineering, other applications (such as rational medium design[10], the elucidation of metabolic and toxicological effects, location of metabolic control[11], determination of maximum

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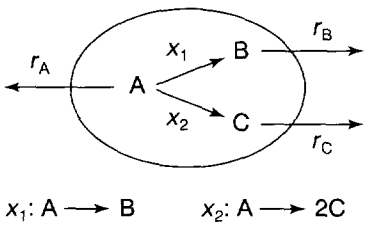
Box 1. Principles of metabolic-flux balancing



Mass balances

over A: $r_A = -3 = -x_1 - x_2$

over B: $r_B = 1 = x_1$

over C: $r_C = 4 = 2x_2$

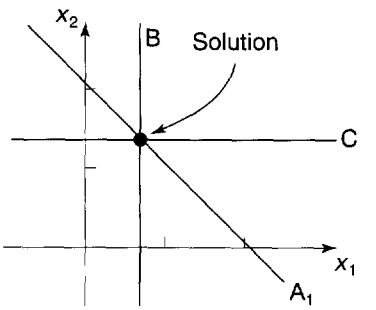


Figure. Principles of metabolic-flux balancing. (Numerical values are chosen arbitrarily.)

Metabolic-flux-balancing techniques are based on relatively simple linear algebra. If the stoichiometry of the relevant intracellular reactions and the cellular composition are known, and the uptake and secretion rates of the relevant metabolites (for example r_A, r_B and r_C in the Fig.) have been measured, the reaction rates (x_1 and x_2 in the Fig.) can be determined using the appropriate mass-balance equations. A reaction network is shown for which one unique solution for the variables x_1 and x_2 can be estimated by least-squares analysis of mass balances A, B and C. The least-squares method, which is used here because there are more constraints (mass balances) than unknowns (fluxes), is calculated by (pseudo)inverting stoichiometric matrix **A** (Ref. 3):

$$\mathbf{A}x = r \Leftrightarrow \mathbf{A}^T\mathbf{A}x = \mathbf{A}^T r \Leftrightarrow x = (\mathbf{A}^T\mathbf{A})^{-1}\mathbf{A}^T r$$

For the stoichiometry and measured metabolic rates given in the figure, this equation reads:

$$\begin{bmatrix} -1 & -1 \\ 1 & 0 \\ 0 & 2 \end{bmatrix} \cdot \begin{bmatrix} x_1 \\ x_2 \end{bmatrix} = \begin{bmatrix} r_A \\ r_B \\ r_C \end{bmatrix} \Leftrightarrow \begin{bmatrix} x_1 \\ x_2 \end{bmatrix} = \frac{1}{9}\begin{bmatrix} 5 & -1 \\ -1 & 2 \end{bmatrix} \cdot \begin{bmatrix} -1 & 1 & 0 \\ -1 & 0 & 2 \end{bmatrix} \cdot \begin{bmatrix} -3 \\ 1 \\ 4 \end{bmatrix} = \begin{bmatrix} 1 \\ 2 \end{bmatrix}$$

This shows that intracellular fluxes can be quantified by measuring only the uptake and secretion rates of the relevant metabolites.

theoretical yields[12] and quantitative prediction of biochemical phenotypes from gene data banks[13]) will all benefit from this computational technique. In this article, the fundamental problem that currently hinders the development of such a method is discussed and various proposed solutions are reviewed. Finally, experiments are suggested to investigate which of these solutions might lead to a method for quantifying metabolic fluxes that uses only mass balances.

Problem definition

Two fundamental problems have been identified in applying this technique to large, complex metabolic networks[3]. First, the estimated flux vector, which is calculated by the least-squares method, may be sensitive to slight perturbations in the measured extracellular rates of the relevant metabolites (r_A, r_B and r_C in Box 1). This sensitivity to error propagation can be checked by calculating the condition number of the system[3,14] (see Glossary), which depends solely on the reaction stoichiometry of the metabolic network. The condition number can be seen to increase with the complexity (Fig. 1a) and the ratio (Fig. 1b) of the stoichiometric coefficients. A large condition number (>100) indicates that the estimated flux distribution is sensitive to measurement errors. Combinations of reactions that cause network sensitivities can be identified using an algorithm developed by Savinell and Palsson[14]. The flow through these reactions either has to be determined independently by isotopic-tracer experiments or removed from the network. However, with a few exceptions[15], the condition number of a metabolic network is generally small (<100), which indicates that error propagation is insignificant[3]. Nevertheless, the condition number of the stoichiometric matrix should be determined prior to least-squares analysis.

A special case of error-sensitive metabolic networks is reaction dependency (Fig. 1c). In contrast to error propagation, this causes observability problems in the determination of the fluxes in the metabolic networks of almost all prokaryotic[9,12,15–18] and eukaryotic[15,19,20] cells. Reaction dependency occurs particularly in cyclic pathways, which are abundantly present in metabolic systems. In Fig. 2a, the problem of a cyclic metabolic pathway is visualized. Reactions in metabolic cycles are linear dependent (see Glossary) and cannot be derived from the extracellular rates. In a metabolic network, such reactions cause singularities, as a result of which the set of mass-balance equations is underdetermined. It is also apparent from Fig. 2 that the mass-balance equations of metabolites A and B yield the same information, as a result of which the fluxes x_1 and x_2 cannot be quantitated solely by

Glossary	
Metabolite (or 'mass') balance	An equation that describes the accumulation and all relevant incoming and outgoing fluxes of a metabolite pool.
Stoichiometric matrix	A matrix that contains information on the reaction stoichiometry of cellular metabolism. The rows and columns of the stoichiometric matrix are associated with the **metabolite balances** and the metabolic fluxes, respectively.
Linear dependency	Metabolite balances are linear dependent if (a linear combination of) the solution planes determined by the **metabolite balances** are parallel. (For example, the mass balances of A and B in Fig. 2 are linear dependent.)
Rank	The maximum number of **linear-independent metabolite balances** in a metabolic network is called the rank of the **stoichiometric matrix**.
Rank deficient	If the **rank** is smaller than the number of metabolic fluxes (the number of rows of the **stoichiometric matrix**), then the metabolic network is rank deficient.
Condition number	The condition number of a **stoichiometric matrix A** (the ratio of the largest to smallest eigenvalue of **A**) is a measure of the sensitivity of the equation $\mathbf{A}x = r$ (Box 1).
Underdetermined networks	Metabolic networks that are **rank deficient** are designated 'underdetermined' to indicate that there are insufficient **linear-independent metabolite balances** to determine the intracellular metabolic fluxes.
Observability	In this context, the extent to which intracellular metabolic fluxes can be determined by the measurement of the extracellular metabolic rates and the biomass composition.
Directionality constraint	The demand that a (number of) flux(es) is non-negative.
Balanceable metabolite	A metabolite whose **mass balance** can be closed.

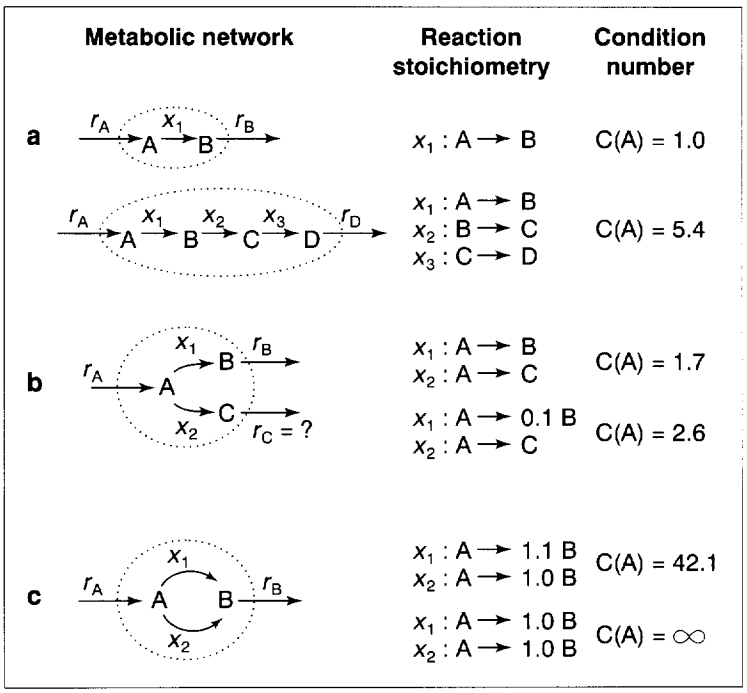


Figure 1

The error sensitivity of a metabolic network is dependent only on the reaction stoichiometry and can be determined by the condition number of the stoichiometric matrix[3]. (**a**) The condition number increases with growing complexity. (**b**) If only r_A and r_B are known, the fluxes x_1 and x_2 can be estimated. It is shown that the condition number increases with the ratio of the stoichiometric coefficients. (**c**) Networks that contain cyclic pathways are error sensitive or not observable [$C(A) = \infty$].

flux-balancing techniques. In this case, the solution space that contains all admissible solutions for fluxes x_1 and x_2 can be visualized by a single line (Fig. 2a). Underdetermined networks such as that shown in Fig. 2a are rank deficient (see Glossary).

Theoretical and experimental solutions

Over the past few years, various approaches have been proposed for the estimation of the fluxes of large, complex metabolic networks by supplementing the underdetermined network with various theoretical assumptions or constraints[3,9,12,15,18–21]. This is particularly important for the determination of fluxes in major metabolic cycles such as the TCA (tricarboxylic acid) cycle, the pentose-phosphate cycle and the malate shunt (the quantification of fluxes in 'futile cycles', where there is no branching of the cycle[22], is not covered in this review). Currently, several research groups are comparing flux distributions estimated on the basis of such assumptions with experimentally determined fluxes using isotopic-tracer methods (Ref. 7; K. Schmidt *et al.*, unpublished). Here, several 'candidate' constraints are reviewed and ways are suggested to find a method for the determination of intracellular fluxes without using isotopic tracers.

Mass balances of cofactors or co-metabolites as additional constraints

When a co-metabolite is produced or consumed in cyclic pathway reactions, the addition of its mass balance may yield a unique solution. In Fig. 2b, it is shown that a least-squares solution exists for a cyclic pathway when the mass balance of metabolite C is added to the network. In reality, addition of the mass balance(s) of co-metabolites is generally not sufficient to generate an (over)determined system. In complex networks, co-metabolites are either produced in more than one cyclic pathway (e.g. carbon dioxide) or are not balanceable (e.g. ATP). In most organisms, carbon dioxide is produced in the pentose cycle, the TCA cycle and the malate shunt and consumed in the pyruvate carboxylase reaction (Fig. 3). Therefore, the

addition of the carbon dioxide balance to the metabolic network will not allow an independent determination of the fluxes in these cycles. Nevertheless, useful information can be obtained from the carbon dioxide production rate, as it provides a means of checking the consistency of the estimated fluxes with respect to the carbon balance.

By addition of the ATP balance to a metabolite network, as shown in Fig. 3, the rank of the stoichiometric matrix increases by one unit, because the ATP balance is linear independent of the other mass balances. As a result, the observability increases and the split ratio of fluxes at either the glucose-6-phosphate or the pyruvate branchpoint can be determined. However, the ATP mass balance cannot be closed, owing to the fact that both ATP yields and ATP requirements for maintenance processes can only be estimated[9,15]. It has been suggested that theoretical calculations of ATP yields result in significantly higher levels than can be deduced from experimental data[23]. Moreover, relatively small changes in such estimates will have large effects on the calculated flux distribution.

The mass balances of reducing equivalents, for example NADH and NADPH, are often used to determine the split ratio of metabolic fluxes at branch points, such as occur in the metabolism at (phosphoenol)pyruvate or glucose-6-phosphate[9,15,17,20]. However, microorganisms interconvert these cofactors with unknown reaction rates using transhydrogenases. Therefore, in order to estimate fluxes in underdetermined networks, NADH and NADPH should be used as a lumped factor to circumvent the problem of transhydrogenase activity[20]. As a consequence, the split ratio of only one branch point (instead of two) can be estimated. Additionally, it has been shown for adipose tissue[19], hybridomas[7] and *Bacillus subtilis*[18] that the estimated flux distribution is very sensitive to the assumptions made or to small changes in the NAD(P)H balance.

Irreversibility of reactions as additional constraints

Some reactions in metabolic networks are considered irreversible. This additional information allows one to set lower boundaries to these particular reactions and to further constrain the solution space, in which all admissible solutions are situated. Although these constraints do not help to overcome observability problems, they can be used to fine tune the unconstrained solution to the equation in Box 1 (Ref. 3), if certain fluxes are negative where they should be irreversible.

An algorithm for the determination of all non-negative, admissible fluxes has been described[24]. This computational method has been applied to describe the basic reaction modes, at the corner points of admissible solution space, in cyclic pathways[25] and to determine optimal flux distributions for the conversion of sugars to aromatic metabolites in *Escherichia coli*[26]. Furthermore, directionality constraints are essential when applying linear optimization techniques, as shown below.

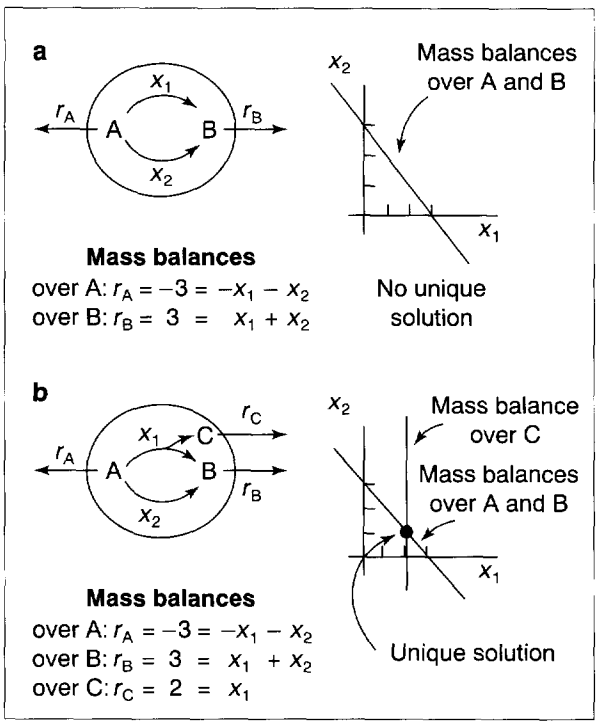


Figure 2

An undetermined (**a**) and a determined (**b**) metabolic network. (**a**) Reactions x_1 and x_2 are linear dependent. (Numerical values are chosen arbitrarily; all reactions are reversible.)

Objective functions as additional constraints; linear optimization

As pointed out above, the mass balances of cofactors such as ATP and NADH may be closed by mere approximation or remain unclosed, as certain biochemical parameters are not quantifiable. Instead, these mass balances can be used to formulate objective functions. Intracellular pools of cofactors for the transfer of energy or reducing power regulate many enzymatic reactions. Therefore a surplus or a lack of cofactors influences the flux distribution of entire metabolic networks. The metabolic pressure from such needs may be translated into linear objective functions and help to estimate optimal solutions that satisfy various metabolic goals[19]. Objective functions that have been applied to estimate fluxes in cyclic routes include, for example, 'maximize NADPH production'[19], 'minimize ATP production'[15] or 'minimize the sum of the squares of fluxes'[20].

Optimal solutions to all admissible flux distributions can be calculated using linear-optimization techniques. The principle of applying these techniques to estimate fluxes in underdetermined metabolic networks is shown in Fig. 4. By definition, linear optimization results in extreme ('optimal') solutions that are the end points of the stoichiometrically feasible domain[2]. In some cases, certain objective functions have no feasible solutions owing to the lack of lower or upper boundaries of cyclic routes. In Fig. 4 for example, this would be the case if the objective function were 'maximize NADH' and if the flux x_1 were *not* constrained instead of being irreversible. Lower

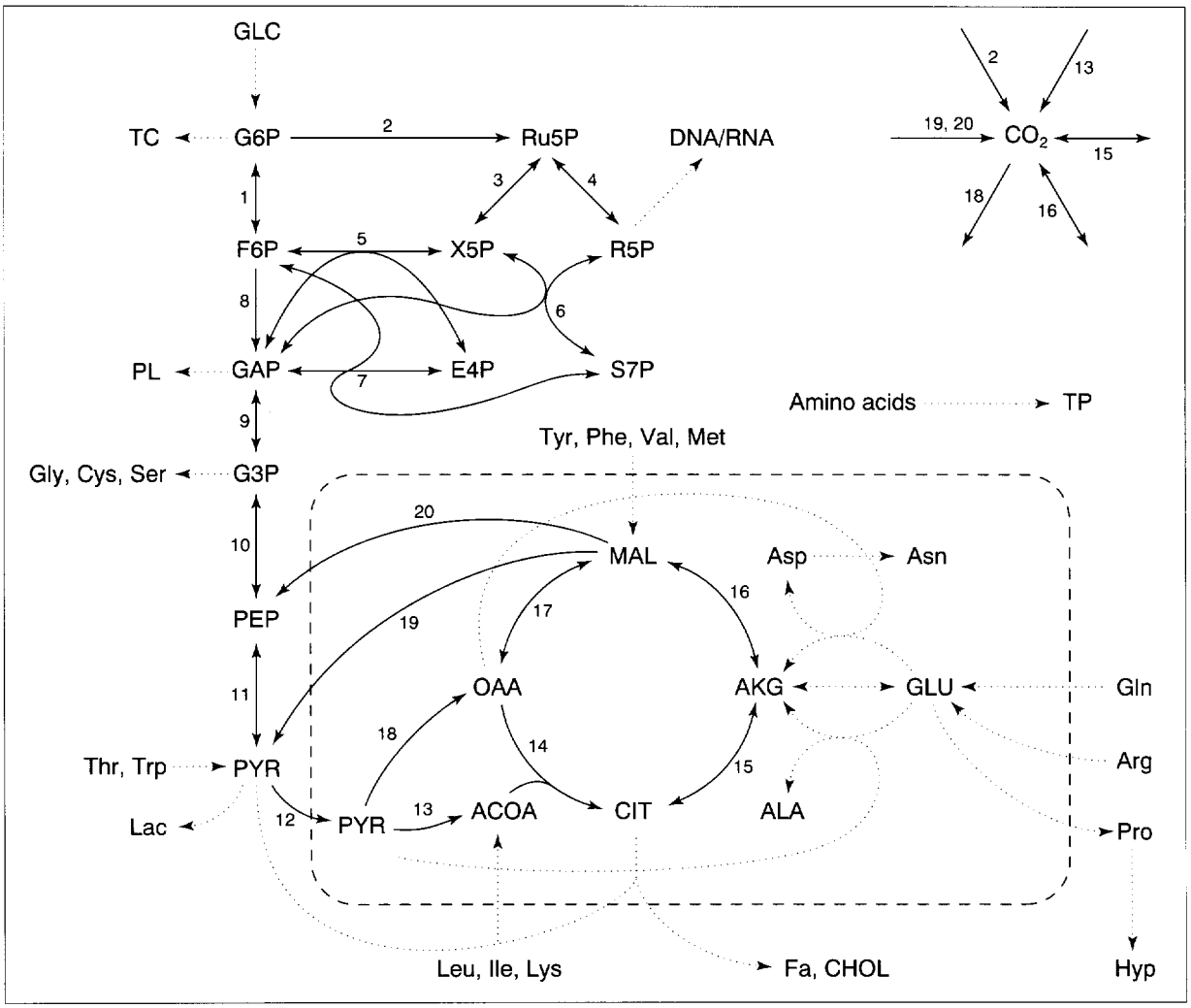


Figure 3

Some metabolic pathways of hybridoma cells[20]. Dashed arrows indicate fluxes that can be quantified using mass balances; solid, numbered arrows represent fluxes that are linear dependent and consequently not calculable using balancing techniques; the dashed line represents the mitochondrial membrane. 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; G3P, 3-phosphoglycerate; LAC, lactate; MAL, malate; OAA, oxaloactetate; 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.

boundaries (which are determined by the irreversibility of fluxes) are essential for obtaining a feasible solution by linear optimization, especially because data for maximum reaction rates are not usually available. However, it is unclear whether the end points of the solution space that is constrained by the mass-balance equations represent the true flux distributions. The answer to this question can be found by isotopic-tracer experiments.

Finding the missing constraint

Little research has been carried out to validate the theoretical assumptions required to determine fluxes in rank-deficient networks by isotopic-tracer methods. It is therefore desirable to compare the flux distributions estimated on the basis of mass balances with the flux distributions determined by labelling experiments for a large range of growth conditions. In this way, a fundamental understanding of the effects of different constraints may be obtained, and it may be determined whether certain theoretical assumptions are generally valid. There are various methods for inducing drastic changes in the primary metabolism of microorganisms. A limitation of important carbon or nitrogen sources or electron acceptors (e.g. oxygen) will force microorganisms to rearrange their flux distribution. Alternatively, an increase in the growth rate, which can be induced by raising the dilution rate of cells in continuous culture, would result in an elevated NADPH requirement for anabolic processes. Theoretically, this will result in higher activity of two cyclic pathways, the pentose-phosphate shunt and the malate shunt. Another possible method for specifically stimulating certain fluxes is the addition of sublethal concentrations of toxic compounds with known effects on metabolism. For example, phenazine methosulphate specifically reoxidizes NADH and NADPH[27], resulting in an increase in NAD(P)H-producing reactions.

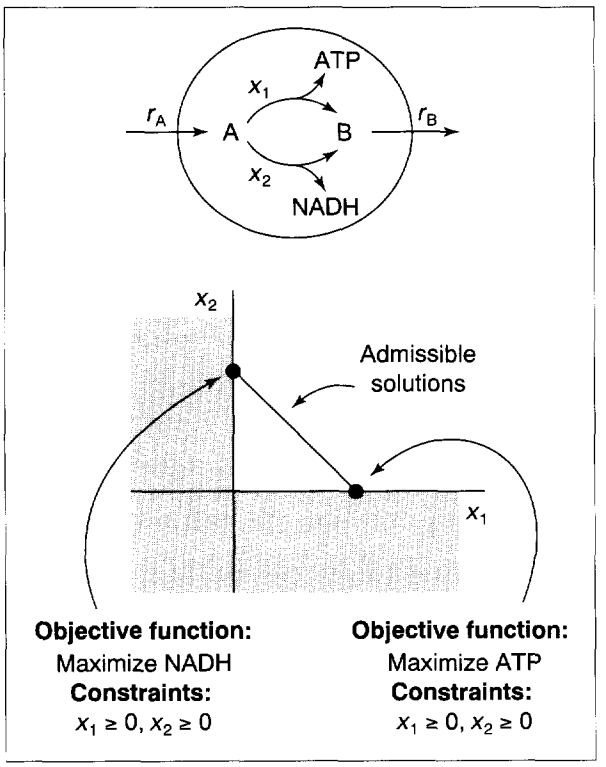


Figure 4
Linear-optimization techniques to estimate fluxes in underdetermined metabolic networks.

In this case, the constraint 'maximize NADH and NADPH production' would theoretically result in an estimated flux distribution that is similar to the experimentally determined fluxes. Similarly, chemical decouplers of oxidative phosphorylation, such as 2,4-dinitrophenol, will lower the activity of the TCA cycle. Fluxes determined from mass balances supplemented with data from isotopic-tracer methods, combined with fluxes estimated from mass balances supplemented with different theoretical constraints, may lead to a fundamental understanding of the validity of the assumptions made previously[9,15,17–20]. In addition, the combined information from mass-balancing techniques and isotopic-tracer experiments will allow the testing of the consistency of both methods, as their combination produces overdetermination of networks. This is relevant, because the use of isotope tracers for the determination of fluxes is an unsolved problem[28].

In addition, there are alternatives that do not require the demanding effort of isotopic-tracer studies and may help to solve the observability problems of rank-deficient metabolic networks. First, various studies have reported quantitative flux data for entire metabolic networks, determined using a set of isotopic tracers[4,29]. From these data (vector x), the extracellular metabolic rates (vector r) can easily be determined using the equation in Box 1. Subsequently, the constraints determined by the mass balances and the metabolic rates can be calculated and various theoretical constraints can be tested for their ability to estimate the fluxes correctly. Second, the presence of certain single-enzyme reactions in metabolic networks causes singularities in the linear set of equations that describes these networks. For example, the pyruvate carboxylase and the transhydrogenase reactions directly cause observability problems. However, in some cases, these enzymes are not active[29], a feature that can be easily measured using enzymatic assays, and if no enzymatic activity is found, then that particular flux can be deleted. Third, for several organisms, the estimated flux distribution, and in particular the split ratio at the glucose-6-phophate branchpoint, has been shown to be extremely sensitive to the NAD(P)H balance[7,18]. As this balance is mainly dependent on the rate of oxygen uptake, an improvement in the accuracy of this measurement may lead to completely observable metabolic networks. Additionally, a weighted least-squares solution could be used to account for uncertainties in mass balances such as the NAD(P)H balance.

Outlook

The near future will show whether investigations (such as some of those suggested here) will lead to the development of a universally valid method for the measurement of intracellular fluxes. In the past, it has been regarded as unwise to make generalizations about the metabolic role of futile cycles because of the lack of available experimental flux data under *in vivo* conditions[22], but this will not be the case for other metabolic cycles such as the pentose shunt, the malate shunt and the TCA cycle. Because metabolic pathways have become targets for genetic and biochemical engineers, the number of isotopic-tracer studies carried out under well-defined conditions will increase. The resulting data may provide a basis for a quantitative understanding of metabolism and help to find missing constraints. However, until then, isotopic-tracer experiments remain indispensable for the quantification of fluxes in cyclic metabolic pathways.

Other applications, such as rational medium design[10], elucidation of metabolic and toxicological effects without using radioactive or stable isotopes, rapid quantitative biochemical studies and the determination of metabolic-control coefficients[11] (for a recent review of the role of metabolic control analysis in rational bioengineering, see Ref. 30) will also benefit from such a breakthrough. In addition, it should be possible to control fermentation processes at the level of intracellular reactions by calculating the metabolic fluxes from on-line measurements of only a few key metabolites. The experimental validation of theoretical constraints will extend our knowledge of the metabolic strategy of various cell lines. Likewise, such constraints can be used as a link between genetics and physiology[13] and thus help to predict phenotypes from genome data banks.

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