J. Math. Biol. 45, 153–181 (2002)
Digital Object Identifier (DOI):
10.1007/s002850200143

Mathematical Biology

S. Schuster · C. Hilgetag · J.H. Woods · D.A. Fell

Reaction routes in biochemical reaction systems: Algebraic properties, validated calculation procedure and example from nucleotide metabolism

Received: 1 January 2001 / Revised version: 17 December 2001 /
Published online: 17 July 2002 –

Abstract. Elementary flux modes (direct reaction routes) are minimal sets of enzymes that can operate at steady state, with all irreversible reactions used in the appropriate direction. They can be interpreted as component pathways of a (bio)chemical reaction network. Here, two different definitions of elementary modes are given and their equivalence is proved. Several algebraic properties of elementary modes are then presented and proved. This concerns, amongst other features, the minimal number of enzymes of the network not used in an elementary mode and the situations where irreversible reactions are replaced by reversible ones. Based on these properties, a refined algorithm is presented, and it is formally proved that this algorithm will exclusively generate all the elementary flux modes of an arbitrary network containing reversible or irreversible reactions or both. The algorithm is illustrated by a biochemical example relevant in nucleotide metabolism. The computer implementation in two different programming languages is discussed.

1. Introduction

The modelling of metabolic pathways has recently attracted ample interest in the light of functional genomics and promising biotechnological applications. The complete sequencing of the genomes of numerous microorganisms has inspired attempts at reconstructing the complete metabolism of these microorganisms (Selkov *et al*., 1997; Dandekar *et al*., 1999; Karp *et al*., 1999; Schilling and Palsson, 2000). In biotechnology, recombinant DNA technology opens up the possibility of redirecting metabolite fluxes towards a desired product or to increase the yield of an existing biotransformation (Liao *et al*., 1996; Stephanopoulos *et al*., 1998). These applications mainly require consideration of structural rather than kinetic properties of biochemical networks. Thus, a number of difficulties can be circumvented because kinetic modelling and simulation in biochemistry are often hampered by the fact

S. Schuster: Department of Bioinformatics, Max Delbrück Centre for Molecular Medicine, D-13092 Berlin-Buch, Germany. e-mail: stschust@mdc-berlin.de

C. Hilgetag: International University Bremen, Campus Ring 1, Rm. 212, D-28759 Bremen, Germany.

J.H. Woods, D.A. Fell: School of Biological and Molecular Sciences, Oxford Brookes University, Headington, Oxford OX3 0BP, UK

Key words or phrases: Convex analysis – Elementary flux modes – Modelling of biochemical systems – Reaction routes – Stoichiometric analysis

that the kinetic properties of enzymes are imperfectly known. There are also discrepancies between *in vitro* values and the *in vivo* behaviour (Benevolensky *et al*., 1994). Furthermore, enzyme activities *in vivo* are subject to frequent changes due to inhibition or activation. In contrast, the structure of biochemical systems in terms of how substances are 'connected' to each other by reactions can be considered constant, unless evolutionary time scales are studied. Therefore, the analysis of the structural invariants of biochemical systems is of particular interest (Reder, 1988; Fell, 1993; Schuster and Hilgetag, 1994; Schilling *et al*., 1999). These include, amongst others, conservation relations (Reder, 1988; Schuster and Höfer, 1991), detailed balanced subnetworks (Schuster and Schuster, 1991), and restrictions on the steady-state fluxes that result from the balance equations (Fell and Small, 1986; Reder, 1988, Schuster and Schuster, 1993).

Reaction routes may be represented by a set of basis vectors in the null-space of the stoichiometry matrix (Horiuti and Nakamura, 1957; Reder, 1988). These vectors are not, however, unique. Moreover, these vectors do not necessarily comply with the irreversibility of some reactions. It is sensible to include information about irreversibility (which largely belongs to the thermodynamic properties of the network as it is related to the equilibrium constants) in the set of structural data because it is independent of enzyme regulation (Seressiotis and Bailey, 1988). If some reactions are irreversible, the region of admissible steady-state flux vectors shrinks to a subset of the null-space. It is of interest to find appropriate basis vectors of this subset, which should preferably be unique. Elaborating on earlier ideas (Clarke, 1981; Leiser and Blum, 1987; Fell, 1990, 1993; Schuster and Schuster, 1993), we introduced the concept of 'elementary flux modes,' which correspond to such unique generating vectors (Schuster and Hilgetag, 1994; Schuster *et al*., 1996). A 'flux mode' is defined as a steady-state flux pattern in which the proportions of fluxes are fixed while their absolute magnitudes are indeterminate. Since we are considering the situation where the kinetic properties of reactions are unknown, we do not concern ourselves with these magnitudes here. A flux mode is called *elementary* if it has a specific simplicity property, which guarantees uniqueness. The concept is related to the concepts of 'direct mechanisms' in chemistry (Milner, 1964; Happel and Sellers, 1982; Sellers, 1984; see also Fishtik and Datta, 2000), biochemical pathways (Seressiotis and Bailey, 1988; Mavrovouniotis *et al*., 1990) and 'minimal T-invariants' in Petri net theory (Starke, 1990). A direct mechanism ('direct path' in the terminology of Milner, 1964) is a minimal set of elementary chemical reaction steps multiplied by appropriate coefficients that when summed up give an overall reaction equation connecting terminal reactants and products (Happel and Sellers, 1982). In a biochemical context, we prefer to avoid the term 'direct mechanism' because elementary modes do not depend on a specific enzyme mechanism such as ping-pong, random, or sequential mechanisms (at least for enzymes with high substrate specificity). In principle, one might use the terms 'direct reaction routes' and 'elementary (flux) modes' interchangeably. While the former term focusses on the topological concept of a path through the system, the latter has the connotation of a flux distribution.

The physiological, medical and biotechnological relevance of determining elementary modes in biochemical networks has been discussed earlier (Schuster and

Hilgetag, 1994; Liao *et al*., 1996; Nuño *et al*., 1997; Schuster *et al*., 1996, 2000; Dandekar *et al*., 1999). In particular, this concept is a useful tool in determining maximal and submaximal yields of biotransformations and in functional genomics. It is also helpful in metabolic flux analysis for determining the calculability of fluxes (Klamt *et al*., 2002).

The present paper is devoted to providing a deeper insight into the mathematics underlying metabolic pathway analysis. In particular, several algebraic properties of elementary modes are given and substantiated in a number of lemmata. For example, an upper bound on the number of enzymes used in an elementary mode is derived. We also give a detailed presentation of an algorithm for detecting all elementary modes to any given biochemical reaction system. It is a refined version of an algorithm outlined earlier (Schuster *et al*., 1996). (In Schuster *et al*., 2000, we have described it for a specific example.) Here, we demonstrate the efficiency of the algorithm in a mathematically rigorous way. To this end, two theorems are proved. In the Discussion section, we will compare our algorithm with several procedures given earlier by other authors for finding biochemical production routes, direct chemical mechanisms, and minimal T-invariants in Petri net theory.

2. Nomenclature and definitions

Consider a biochemical reaction network with r reactions. As often done in biochemical modelling, we make a distinction between internal and external metabolites. The former (say, n in number) have variable concentrations, while the latter (also called source and sink species) have concentrations assumed to be constant, for example due to regulated supply. Let $\mathbf{N}$ denote the stoichiometry matrix of the system and η be its rank (cf. Reder, 1988; Heinrich and Schuster, 1996). $\mathbf{I}$ and $\mathbf{0}$ stand for the identity matrix and null matrix, respectively, of suitable dimensions. Consider, for example, the system depicted in Fig. 1. Its stoichiometry matrix reads

$$\mathbf{N} = \begin{pmatrix} 1 & -1 & 0 & -1 \\ 0 & 1 & -1 & 0 \end{pmatrix}. \tag{2.1}$$

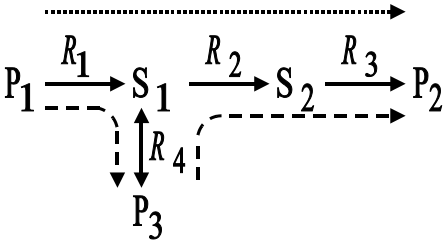

Fig. 1. Simple reaction system illustrating the difference between convex basis and elementary modes. The two flux distributions indicated by dashed arrows correspond to the vectors $\mathbf{f}^{(1)}$ and $\mathbf{f}^{(2)}$ forming the convex basis. The additional elementary mode is represented by a dotted arrow. P_i, external metabolites; S_i, internal metabolites. The reaction with double arrowheads is assumed to be reversible.

Many biochemical systems attain an asymptotically stable steady state. In such a state, the vector of reaction rates, $\mathbf{V}$, fulfils the condition

$$\mathbf{NV} = \mathbf{0}. \tag{2.2}$$

The rows of $\mathbf{N}$ are to correspond to the internal metabolites only. If also the external species are taken into account in this matrix, care must be taken as the steady-state condition (2.2) only applies to the internal metabolites. The complete set of vectors $\mathbf{V}$ obeying Eq. (2.2) defines a region called the null-space of $\mathbf{N}$ (cf. Groetsch and King, 1988). Its dimension equals $r - \eta$ (r, number of reactions).

We decompose the vector of fluxes, $\mathbf{V}$, into two subvectors, $\mathbf{V}_{\text{rev}}$ and $\mathbf{V}_{\text{irr}}$, which include the fluxes of the reversible and irreversible reactions, respectively. Irreversibility is not meant to exclude a reverse step, but this step should always have a lower rate than the forward step. Without loss of generality, we can define the orientation of reactions so that

$$\mathbf{V}_{\text{irr}} \geq \mathbf{0}. \tag{2.3}$$

In accordance with relation (2.3), we number the reactions so that $\mathbf{N}$ can be partitioned as

$$\mathbf{N} = (\mathbf{N}_{\text{rev}} \;\; \mathbf{N}_{\text{irr}}). \tag{2.4}$$

The vector inequality (2.3) implies that only part of the null-space of $\mathbf{N}$ is an admissible region for the flux vector. Any particular inequality, $v_i \geq 0$, included in relation (2.3) together with Eq. (2.2), defines a half-space in the null-space of $\mathbf{N}$. The intersection of all these half-spaces determines what is called in mathematics a convex polyhedral cone, which we will refer to as the flux cone, $\boldsymbol{F}$. Fig. 2 shows the flux cone for the system depicted in Fig. 1. (For examples of three-dimensional flux cones, see, e.g., Fig. 2 in Schuster and Schuster, 1993, or Fig. 2 in Schilling *et al.*, 1999). For analysing convex polyhedral cones, we need mathematical tools from convex analysis (cf. Rockafellar, 1970), which is more complex than linear algebra because inequality constraints have to be considered. The flux cone, $\boldsymbol{F}$, is convex because if any two vectors $\mathbf{V}^{(1)}$ and $\mathbf{V}^{(2)}$ satisfy Eqs. (2.2) and (2.3), so does any convex combination of these vectors, that is, any linear combination $\lambda\mathbf{V}^{(1)} + (1-\lambda)\mathbf{V}^{(2)}$ with $0 \leq \lambda \leq 1$. The cone $\boldsymbol{F}$ has, moreover, the property that if any vector $\mathbf{V}$ lies in it, so does any positive multiple $\mu\mathbf{V}$ ($\mu > 0$). For some vectors, this property even holds for any real μ (positive or negative). Accordingly, there are a set of vectors $\mathbf{f}_k$ and a set of vectors $\mathbf{b}_i$ which span the cone:

$$\boldsymbol{F} = \left\{ \mathbf{V} \in \mathbb{R}^r : \mathbf{V} = \sum_k \lambda_k \mathbf{f}_k + \sum_i \beta_i \mathbf{b}_i,\, \lambda_k,\, \beta_i \in \mathbb{R},\, \lambda_k \geq 0 \right\}. \tag{2.5}$$

It is of interest to detect an appropriate set of such generating vectors. The following two cases can be distinguished:

(a) Cone $\boldsymbol{F}$ is pointed, that is, any two vectors belonging to $\boldsymbol{F}$ make an angle of less than 180 degrees. In other words, there is no vector $\mathbf{b}_i$ belonging to $\boldsymbol{F}$ for which also $-\mathbf{b}_i$ lies in $\boldsymbol{F}$. This case occurs, in particular, when all reactions are irreversible

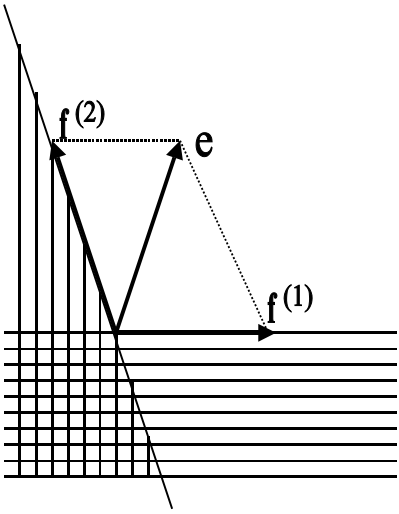

Fig. 2. Geometrical representation of the elementary modes, $\mathbf{f}^{(1)}$, $\mathbf{f}^{(2)}$ and $\mathbf{e}$, for the reaction scheme shown in Fig. 1. The plane spanned by the vectors $\mathbf{f}^{(1)}$ and $\mathbf{f}^{(2)}$ has been chosen to be the drawing plane. In the horizontally shaded area, v_2 and v_3 would be negative. In the vertically shaded area, v_1 would be negative.

because $\mathbf{b}_i$ and $-\mathbf{b}_i$ cannot both use the irreversible reactions in the right direction. However, this case can also occur when several reactions are reversible.

It has been shown in convex analysis (Rockafellar, 1970) that pointed convex polyhedral cones are spanned by a finite set of generating vectors which are unique up to scalar multiples. These vectors lie on the edges of the cone.

(b) Cone $\boldsymbol{F}$ is not pointed, that is, it contains a vector $\mathbf{b}_i$ belonging to $\boldsymbol{F}$ for which also $-\mathbf{b}_i$ lies in $\boldsymbol{F}$. An example is provided by a three-dimensional half-space. This half-space can be spanned by two vectors $\mathbf{b}_i$ lying in the plane confining the half-space and one vector $\mathbf{f}_k$ pointing into the interior of the half-space. Obviously, neither $\mathbf{b}_i$ nor $\mathbf{f}_k$ are uniquely determined because they do not lie on any edge. In what follows we will show how we can nevertheless find a unique set of generating vectors.

For the further presentation, the following symbol will turn out to be helpful. For any flux vector $\mathbf{V}$ with elements v_k, let

$$\mathrm{S}(\mathbf{V}) = \{i : v_i = 0\}. \tag{2.6}$$

The complement of this set, i.e. the set $\{1, 2, \ldots, r\}\backslash\mathrm{S}(\mathbf{V})$ is called the support of $\mathbf{V}$ (cf. Starke, 1990; Colom and Silva, 1990). Now we give two central definitions.

Definition 1. *A flux mode, $\boldsymbol{M}$, is defined as the set*

$$\boldsymbol{M} = \{\mathbf{V}\in\mathbb{R}^r\colon \mathbf{V} = \lambda\mathbf{V}^*, \lambda > 0\}, \tag{2.7}$$

where $\boldsymbol{V}^*$ is an r-vector (unequal to the null vector) fulfilling the following two conditions:

(C1) *Steady-state condition, i.e. Eq. (2.2).*
(C2) *Sign restriction:* $\mathbf{V}^*$ *contains a subvector,* $\mathbf{V}^*_{\text{irr}}$*, that fulfils inequality (2.3), with the components of* $\mathbf{V}^*_{\text{irr}}$ *corresponding to the irreversible reactions.*

Definition 2. *A flux mode* $\boldsymbol{M}$ *with a representative* $\mathbf{V}^*$ *is called an elementary (flux) mode if, and only if,* $\mathbf{V}^*$ *fulfils the condition*
(C3) *Non-decomposability.* $\mathbf{V}^*$ *cannot be represented as a positive linear combination,*

$$\mathbf{V}^* \neq \lambda_1 \mathbf{V}' + \lambda_2 \mathbf{V}''; \quad \lambda_1, \lambda_2 > 0, \tag{2.8}$$

of two flux vectors $\mathbf{V}'$ *and* $\mathbf{V}''$ *(unequal to the null vector) that have the properties:*

(i) $\mathbf{V}'$ *and* $\mathbf{V}''$ *themselves obey restrictions (C1) and (C2),*
(ii) *both* $\mathbf{V}'$ *and* $\mathbf{V}''$ *contain zero elements wherever* $\boldsymbol{V}^*$ *does, and they include at least one additional zero component each,*

$$\mathrm{S}(\mathbf{V}^*) \subset \mathrm{S}(\mathbf{V}'), \quad \mathrm{S}(\mathbf{V}^*) \subset \mathrm{S}(\mathbf{V}''). \tag{2.9a,b}$$

In what follows, for simplicity's sake, elementary modes will be denoted by suitable representative vectors. Condition (C3) is related to the property of generating vectors of pointed cones saying that they cannot be decomposed into two other vectors belonging to the cone. In the case of non-pointed cones, some vectors pointing into the interior of the cone are needed to complete a set of generating vectors. Although they are decomposable into other vectors, they can be chosen so as to be non-decomposable into simpler vectors, with the property of simplicity defined via the positions of zeros (cf. Condition C3,ii). The latter implies that decomposition of a mode into two others must not involve additional enzymes (Seressiotis and Bailey, 1988). An alternative, equivalent formulation of condition (C3) will be given in Section 3.

In convex analysis, algorithms were developed for computing sets of generating vectors of convex cones (Chernikov, 1968; Nožička *et al*., 1974). There is, however, relatively little literature about this topic. The algorithm given by Nožička *et al*. (1974) and the two, more difficult, algorithms proposed by Chernikov (1968) yield a minimum set of generating vectors in the sense that they span the cone while any subset of it does not. If all reactions are irreversible, this set corresponds to all edges of the cone. Moreover, in this case, it represents a complete set of elementary modes, as we have shown earlier (Schuster and Hilgetag, 1994).

In the presence of reversible reactions, however, the above-mentioned algorithms do not normally yield all modes fulfilling Definition 2. It is certainly an option to restrict oneself to a minimum set of generating vectors (called convex basis in Pfeiffer *et al*., 1999), as was done by Nuño *et al*. (1997) and Schilling *et al*. (2000). On the other hand, this set is often not unique. This arbitrariness is unsatisfactory because we do not know which generating vectors are of biological importance. Several authors (Clarke, 1981; Seressiotis and Bailey, 1988; Liao *et al*., 1996; Schilling *et al*., 2000) proposed solving the problem of non-uniqueness of the convex basis by decomposing several or all reversible reactions into forward and reverse steps, which are irreversible. This has, however, the drawback that spurious cyclic modes consisting of the forward and reverse steps of one and the same

reaction have to be cancelled after computation of the pathways (see Discussion section for a more detailed reasoning on this point).

Another drawback of the convex basis is that the vectors belonging to this basis often do not represent all biochemically relevant pathways in a network. For example, it can easily be seen that, for the reaction scheme shown in Fig. 1, the flux vectors $\mathbf{V} = \mathbf{f}^{(1)} = (1 \quad 0 \quad 0 \quad 1)^{\mathrm{T}}$ and $\mathbf{V} = \mathbf{f}^{(2)} = (0 \quad 1 \quad 1 \quad -1)^{\mathrm{T}}$ form a convex basis; that is, all admissible flux distributions at steady state can be written as a non-negative linear combination of these two vectors. Moreover, these vectors represent elementary flux modes according to Definition 2. However, there is another elementary mode, which can be written as $\mathbf{e} = (1 \quad 1 \quad 1 \quad 0)^{\mathrm{T}}$. Although it is the sum of $\mathbf{f}^{(1)}$ and $\mathbf{f}^{(2)}$, there are good reasons for considering this flux distribution as equally important as $\mathbf{f}^{(1)}$ and $\mathbf{f}^{(2)}$. For example, the reaction sequence from P_1 to P_2 could be conceived of as a simple model of glycolysis or an amino acid synthesis pathway. From these pathways, usually side routes branch off. In glycolysis, for example, fructose 6-phosphate can be either withdrawn or fed in by the pentose phosphate pathway depending on physiological conditions. If we restricted the definition of direct reaction routes to the convex basis, glycolysis would not be obtained as such a pathway because it is then the sum of two pathways that use this side reaction in opposite directions. With Definition 2, however, glycolysis results as an elementary mode (cf. Schuster *et al.*, 2000).

3. Properties of elementary modes

As stated in the following lemma, elementary modes have the important property that each admissible flux distribution can be expressed as a superposition of such modes. For example, each vector lying in the flux cone $\boldsymbol{F}$ shown in Fig. 2 can be expressed as a non-negative linear combination of the vectors $\mathbf{f}^{(1)}$ and $\mathbf{f}^{(2)}$ and, optionally, the vector $\mathbf{e}$.

Lemma 1. *All vectors* $\mathbf{V}$ *fulfilling conditions (C1) and (C2) either represent elementary modes or are positive linear combinations of vectors representing elementary modes,*

$$\mathbf{V} = \sum_l \eta_l \mathbf{m}_l, \quad \eta_l > 0, \tag{3.1}$$

where the sum runs over at least two different indices l, and all $\mathbf{m}_l$ *have zero components wherever* $\mathbf{V}$ *has zero components and include at least one additional zero each,*

$$\mathrm{S}(\mathbf{V}) \subset \mathrm{S}(\mathbf{m}_l). \tag{3.2}$$

Furthermore, all those $\mathbf{m}_l$ *that enter Eq. (3.1) represent reversible elementary modes if, and only if,* $\mathbf{V}$ *represents a reversible flux mode.*

Proof. Assume there is a vector $\mathbf{V}$ fulfilling the steady-state condition (C1) and sign restriction (C2) that does not represent an elementary mode itself and cannot be decomposed as in Eq. (3.1). $\mathbf{V}$ then would not fulfil condition (C3), so that it could be decomposed into two steady-state flux vectors,

$$\mathbf{V} = \eta'\mathbf{V}' + \eta''\mathbf{V}''; \quad \eta', \eta'' > 0 \tag{3.3}$$

with $S(\mathbf{V}) \subset S(\mathbf{V}')$ and $S(\mathbf{V}) \subset S(\mathbf{V}'')$. If $\mathbf{V}'$ and $\mathbf{V}''$ stand for elementary modes, the assumption of the indirect proof is falsified. If not, $\mathbf{V}'$ or $\mathbf{V}''$ or both could be further decomposed, and so forth. Since this consecutive decomposition implies a steady increase in the number of zero components, it would eventually end with a set of vectors that cannot be split any further, i.e. with a set of representatives of elementary modes satisfying relation (3.2).

If all $\mathbf{m}_l$ entering Eq. (3.1) represent reversible modes, all of their components corresponding to irreversible reactions are zero. Therefore, also $\mathbf{V}$ stands for a reversible mode. Assume now that $\mathbf{V}$ represents a reversible flux mode and that one $\mathbf{m}_l$ entering Eq. (3.1) represents an irreversible elementary mode. Then one component of $\mathbf{m}_l$, m_{li}, that corresponds to an irreversible reaction, would be positive and, hence, so would v_i because no component m_{ki} of any other mode can be negative. This is a contradiction of the fact that $-\mathbf{V}$ needs to represent a flux mode. This completes the proof.

Lemma 2. *For any pair of vectors,* $\mathbf{V}^*$ *and* $\mathbf{V}^{**}$*, with* $\mathbf{V}^*$ *representing an elementary flux mode and* $\mathbf{V}^{**}$ *representing a flux mode and having zero components wherever* $\mathbf{V}^*$ *has zero components:*

$$S(\mathbf{V}^*) \subseteq S(\mathbf{V}^{**}), \tag{3.4}$$

$\mathbf{V}^{**}$ *either represents the same elementary mode as* $\mathbf{V}^*$ *or the same elementary mode as* $-\mathbf{V}^*$*, which implies* $S(\mathbf{V}^*) = S(\mathbf{V}^{**})$.

Proof. Let p denote the number of indices i for which $v_i^* \neq 0$ and q the number of indices j for which $v_j^{**} \neq 0$. We apply the method of induction with respect to p. If $p = 1$, then also q equals 1, so that the lemma obviously holds true.

Now assume that Lemma 2 holds true for all p with $p \leq k$. If $p = k + 1$, we consider two cases according to whether or not there is an index l with

$$\operatorname{sgn}(v_l^*) = -\operatorname{sgn}(v_l^{**}) \neq 0. \tag{3.5}$$

In the former case, we calculate a linear combination for which $v_l' = 0$ (see Fig. 3A):

$$\mathbf{V}' = \left|v_l^{**}\right| \mathbf{V}^* + \left|v_l^*\right| \mathbf{V}^{**}. \tag{3.6}$$

$\mathbf{V}'$ fulfils conditions (C1) and (C2). Owing to relation (3.4) and, $v_l' = 0$, $\mathbf{V}'$ contains zero elements wherever $\mathbf{V}^*$ does, and at least one additional zero component. Thus, the number of non-zero components of $\mathbf{V}'$ is less than, or equal to, k. So we can apply the induction hypothesis to $\mathbf{V}'$ and $\mathbf{V}^*$, which ensures $\mathbf{V}^*$ is a multiple of $\mathbf{V}'$ and, hence, also of $\mathbf{V}^{**}$.

If there is no index l fulfilling relation (3.5), we define

$$\rho = \min_i \left(v_i^*/v_i^{**}\right) \quad \text{for all } i \text{ with } \operatorname{sgn} v_i^* = \operatorname{sgn} v_i^{**} \neq 0. \tag{3.7}$$

Let h be an index with $v_h^*/v_h^{**} = \rho$. The vector $\mathbf{V}^* - \rho\mathbf{V}^{**}$ obviously fulfils the steady-state condition (2.2) and its h-th component is zero (Fig. 3B). Consider now all indices j corresponding to irreversible reactions. For these indices, we have $v_j^* > 0$ and $v_j^{**} > 0$. Eq. (3.7) implies that

$$v_j^* - \rho v_j^{**} \geq v_h^* - \rho v_h^{**} = 0. \tag{3.8}$$

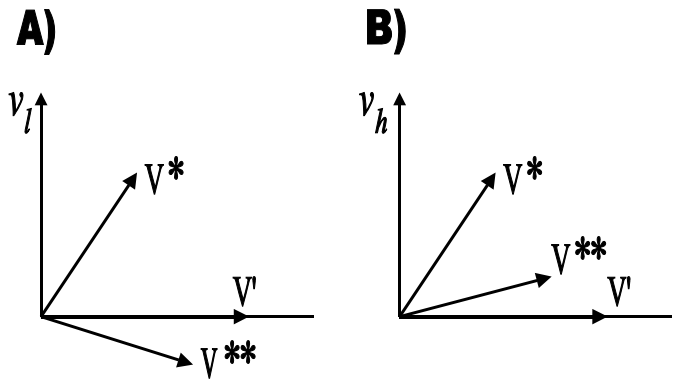

Fig. 3. Schematic picture of the vectors considered in the proof to Lemma 2, for the case where there is an index l fulfilling relation (3.5) (A) and in the case where there is no such index (B). The horizontal lines represent the hyperplanes in which $v_l = 0$ (A) and $v_h = 0$ (B), respectively.

The vector $\mathbf{V}^* - \rho\mathbf{V}^{**}$ therefore satisfies condition (C2) irrespective of whether reaction h is irreversible. It is, hence, an admissible steady-state flux vector, unless it equals the null vector, in which case Lemma 2 obviously holds true. Owing to relation (3.4) and $v_h^* - \rho v_h^{**} = 0$ (cf. Eq. (3.8)), the number of non-zero components of $\mathbf{V}^* - \rho\mathbf{V}^{**}$ is less than, or equal to, k. So the induction hypothesis ensures $\mathbf{V}^*$ is a multiple of $\mathbf{V}^* - \rho\mathbf{V}^{**}$ and, hence, also of $\mathbf{V}^{**}$. This completes the proof.

Lemma 2 may be used to phrase condition (C3) in Definition 2 in a different, yet equivalent way:

(C3′) Simplicity. There exists no vector, $\mathbf{V}^{**}$ (unequal to the null vector) with the properties

(i) $\mathbf{V}^{**}$ obeys conditions (C1) and (C2),

(ii) $\mathbf{V}^{**}$ contains zero components wherever $\mathbf{V}^*$ does and in at least one additional position,

$$\mathrm{S}(\mathbf{V}^*) \subset \mathrm{S}(\mathbf{V}^{**}). \tag{3.9}$$

This means that an elementary mode involves a minimal number of reactions. ‘Minimal’ is meant to imply that if a reaction is omitted from the reaction route, then there is no way to eliminate all of the internal metabolites by linearly combining the remaining reactions. Moreover, it can be concluded that each elementary mode has the property that every other reaction route must involve at least one reaction not present in the elementary mode under consideration.

The equivalence of conditions (C3) and (C3′) can be shown as follows. Any flux mode satisfying condition (C3) also fulfils condition (C3′) due to Lemma 2. Assume that there is a flux mode satisfying condition (C3′) which does not fulfil condition (C3). It can then be decomposed into two modes which involve more zero components (cf. Eq. (3.3)). This is a contradiction of condition (C3′).

Condition (C3′) is similar to conditions used in the definitions of direct mechanisms (Sellers, 1984; Mavrovouniotis, 1992) and minimal T-invariants (Starke, 1990; Colom and Silva, 1990). Whereas condition (C3) can be interpreted in terms of a decomposition of flux patterns, condition (C3′) corresponds to an interpretation of elementary modes as those sets of reactions that keep proceeding after inhibition of a number of enzymes, such that inhibition of a further, still active, enzyme leads to cessation of any non-zero flux.

If we assume that reaction R_4 in Fig. 1 is irreversible in the direction of consumption of P_3, then the reaction route producing P_3 must be discarded. This is a special case of the following

Lemma 3. *For two reaction systems* Σ_a *and* Σ_b *differing only in that some reactions are reversible in* Σ_b *while being irreversible in* Σ_a*, all elementary modes of* Σ_a *are also elementary modes in* Σ_b*, which may involve additional elementary modes.*

Proof. Suppose there is an elementary mode, $\mathbf{V}^*$, in Σ_a that is not an elementary mode in Σ_b. $\mathbf{V}^*$ would nevertheless fulfil Eq. (2.2) and relation (2.3) for Σ_b, because the stoichiometric matrix is the same in both systems and relation (2.3) is less strict for Σ_b than it is for Σ_a. Therefore, $\mathbf{V}^*$ would be an admissible flux distribution in Σ_b. As $\mathbf{V}^*$ is assumed not to be elementary in Σ_b, Lemma 2 implies that there is a vector $\mathbf{V}^{**}$ fulfilling relation (3.9) for subsystem Σ_b. If this mode, $\mathbf{V}^{**}$, were not only admissible in Σ_b, but also in Σ_a, $\mathbf{V}^*$ would not be elementary in Σ_a, a contradiction. Therefore, $\mathbf{V}^{**}$ must violate the sign restriction (2.3) for at least one reaction that is irreversible in Σ_a and reversible in Σ_b. Let k be the index of this reaction. However, due to relation (3.9), k must be a position where $\mathbf{V}^*$ has a non-zero component. More exactly, v_k^* must be positive because v_k^{**} violates the irreversibility condition. We can now combine $\mathbf{V}^*$ and $\mathbf{V}^{**}$ according to

$$\hat{\mathbf{V}} = v_k^* \mathbf{V}^{**} + \left| v_k^{**} \right| \mathbf{V}^*. \tag{3.10}$$

This is a non-negative linear combination and does not, hence, violate condition (2.3) in Σ_a for index k. If there is an index, k', for which $\hat{v}_{k'}$ violates condition (2.3), then we can calculate a linear combination of $\mathbf{V}^*$ and $\hat{\mathbf{V}}$ analogous to Eq. (3.10). Repeating this until no index violating condition (2.3) is left, we arrive at a vector that has zero components wherever $\mathbf{V}^*$ does and an additional zero component (at position k). Therefore, Lemma 2 implies that $\mathbf{V}^*$ cannot be elementary in Σ_a, a contradiction. This completes the proof.

As stated above, elementary modes are minimal with respect to the enzymes involved. The question arises as to how many enzymes are usually involved in an elementary mode. Consider again the scheme in Fig. 1. The null-space of the stoichiometry matrix to this system has dimension two; that is, the system has two degrees of freedom with respect to flux. On the other hand, the three elementary flux modes have the property that each of them contains at least one zero component. These two properties are interrelated for any reaction system, as expressed by the following

Lemma 4. *Any vector representing an elementary mode involves at least* $\gamma - 1$ *zero components, with* $\gamma = r - \eta$ *denoting the dimension of the null-space of* $\mathbf{N}$.

Proof. Suppose that, in a given reaction system Σ_a, there is an elementary mode $\mathbf{V}^*$ involving fewer than $\gamma - 1$ zeros. Now consider the hypothetical situation that all reactions in the system are reversible (system Σ_b). Lemma 3 implies that the elementary mode under consideration is also elementary in Σ_b. If all reactions are reversible, all vectors lying in the null-space of $\mathbf{N}$ are admissible flux vectors. Therefore, Lemma 1 implies that there are at least γ elementary modes because, otherwise, the admissible flux region could not be spanned by these modes. According to the hypothesis of the indirect proof, $\mathbf{V}^*$ would involve more than $r-\gamma+1 = \eta+1$ non-zero components. We now choose any $\eta+1$ of these components. Let I denote the set of subscripts of these components. The members of any set of $\eta+1$ columns of $\mathbf{N}$ are linearly dependent on each other (for example, any three columns in the matrix given in Eq. (2.1) are linearly dependent). Accordingly, we can find non-zero numbers α_j such that $\sum_{j\in I} n_{ij}\alpha_j = 0$ for all i. Therefore, a vector $\mathbf{V}^{**}$ satisfying $v_j^{**} = \alpha_j$ for $j \in I$ and $v_j^{**} = 0$ otherwise represents a flux mode. Applying Lemma 2 for system Σ_b, we see that $\mathbf{V}^*$ cannot be an elementary mode, a contradiction.

It is worth noting that Milner (1964) has shown, in a different way and using the tacit assumption that all intermediates be linearly independent (i.e., that there is no conservation relation among them) that the number of intermediates plus one is the maximum number of steps that can occur in a direct path. Lemma 4 is more general in that the existence of irreversible reactions is allowed.

4. The algorithm

As explained at the end of Section 2, we advocate extending the convex basis by including flux modes that are elementary as well in the sense of Definition 2. To derive an algorithm for computing the complete set of reversible and irreversible elementary modes in reaction networks of any complexity, we have modified the algorithm of Nožička *et al.* (1974) in the following way. Basically, one seeks special solutions to a system of linear homogeneous equations and inequalities.

1. Start with the tableau

$$\mathbf{T}^{(0)} = \begin{pmatrix} \mathbf{B}^{(0)} \\ \mathbf{F}^{(0)} \end{pmatrix} = \begin{pmatrix} \mathbf{N}_{\text{rev}}^{\text{T}} & \mathbf{I} & \mathbf{0} \\ \mathbf{N}_{\text{irr}}^{\text{T}} & \mathbf{0} & \mathbf{I} \end{pmatrix}. \tag{4.1}$$

In implementing the algorithm on computer, one can circumvent the renumbering of reactions preceding the decomposition (2.4), by starting from the tableau $\mathbf{T}^{(0)} = (\mathbf{N}^{\text{T}}\ \mathbf{I})$ and rearranging its rows so that the rows of $\mathbf{N}_{\text{rev}}^{\text{T}}$ stand next to each other. Instead of the identity matrix on the right-hand side of $\mathbf{T}^{(0)}$, one then has a matrix produced from the identity matrix by rearrangement of rows, that we designate for later use as in

$$\mathbf{T}^{(0)} = \begin{pmatrix} \mathbf{N}_{\text{rev}}^{(0)^{\text{T}}} & \mathbf{M}_{\text{rev}}^{(0)} \\ \mathbf{N}_{\text{irr}}^{(0)^{\text{T}}} & \mathbf{M}_{\text{irr}}^{(0)} \end{pmatrix}. \tag{4.2}$$

2. From the tableau $\mathbf{T}^{(j)}(j = 0, 1, 2, \ldots, n-1)$, the elements of which are denoted by $t_{i,h}^{(j)}$, calculate the next tableau, $\mathbf{T}^{(j+1)} = (\mathbf{N}^{(j+1)^{\text{T}}}\ \mathbf{M}^{(j+1)})$ in the following way:

a) For each row of $\mathbf{M}^{(j)}$, determine the set $\mathrm{S}(\mathbf{m}_i^{(j)})$ recording the position of the zeroes, as defined in Eq. (2.6).

b) First, all rows of $\mathbf{T}^{(j)}$ with a zero in the $(j+1)$th column of $\mathbf{B}^{(j)}$ are copied into $\mathbf{T}^{(j+1)}$. Then, new rows formed by allowed linear combinations of pairs of rows of $\mathbf{B}^{(j)}$ consecutively go into $\mathbf{T}^{(j+1)}$ if they fulfil the conditions

$$t_{i,j+1}^{(j)} \cdot t_{m,j+1}^{(j)} \neq 0, \tag{4.3}$$

$$\mathrm{S}\left(\mathbf{m}_i^{(j)}\right) \cap \mathrm{S}\left(\mathbf{m}_m^{(j)}\right) \not\subseteq \mathrm{S}\left(\mathbf{m}_l^{(j+1)}\right) \tag{4.4}$$

for all row indices l belonging to the tableau $\mathbf{B}^{(j+1)}$ as it has been compiled until that moment. If this tableau does not yet contain any row, condition (4.4) is taken to be fulfilled. The combination of rows should be done so as to give a zero element in the $(j+1)$th column,

$$\boldsymbol{\theta} = t_{i,j+1}^{(j)} \cdot \mathbf{t}_m^{(j)} - t_{m,j+1}^{(j)} \cdot \mathbf{t}_i^{(j)}. \tag{4.5}$$

Relation (4.4) implies that combination of the rows will lead to a new row containing a set of zeroes not yet generated.

If any pair of rows $\mathbf{t}_i^{(j)}$ and $\mathbf{t}_m^{(j)}$ pass condition (4.4) and are combined and added to the tableau, all the rows previously added to the new tableau must be checked to ensure that

$$\mathrm{S}\left(\mathbf{m}_l^{(j+1)}\right) \not\subset \mathrm{S}\left(\mathbf{m}_i^{(j)}\right) \cap \mathrm{S}\left(\mathbf{m}_m^{(j)}\right). \tag{4.6}$$

If this condition is not fulfilled for some index l, $\mathbf{t}_l^{(j+1)}$ has to be cancelled in $\mathbf{B}^{(j+1)}$.

c) The first part of $\mathbf{F}^{(j+1)}$ is constructed by using all rows of $\mathbf{F}^{(j)}$ with $t_{i,j+1}^{(j)} = 0$. Now, transfer into $\mathbf{F}^{(j+1)}$ appropriate linear combinations of all pairs of rows, $\mathbf{t}_i^{(j)}$ and $\mathbf{t}_m^{(j)}$, of $\mathbf{F}^{(j)}$ containing non-zero elements of opposite sign in the $(j+1)$th column, that is,

$$t_{i,j+1}^{(j)} \cdot t_{m,j+1}^{(j)} < 0 \tag{4.7}$$

and also satisfying relation (4.4) for all row indices l belonging to $\mathbf{F}^{(j+1)}$ as it has been compiled until that moment. Appropriate combination means addition after scaling to ensure the $(j+1)$th column of the result will be zero, that is,

$$\boldsymbol{\phi} = \left|t_{i,j+1}^{(j)}\right| \cdot \mathbf{t}_m^{(j)} + \left|t_{m,j+1}^{(j)}\right| \cdot \mathbf{t}_i^{(j)}. \tag{4.8}$$

When condition (4.4) is passed, also condition (4.6) has to be tested and any row $\mathbf{t}_l^{(j+1)}$ failing this check has to be cancelled in $\mathbf{F}^{(j+1)}$.

d) Similarly, for all pairs of rows, $\mathbf{t}_i^{(j)}$ and $\mathbf{t}_m^{(j)}$, with $\mathbf{t}_i^{(j)}$ belonging to $\mathbf{B}^{(j)}$ and $\mathbf{t}_m^{(j)}$ belonging to $\mathbf{F}^{(j)}$ and fulfilling relations (4.3) and (4.4) for all row indices l belonging to $\mathbf{F}^{(j+1)}$ as it has grown until that moment, calculate the vectors

$$\boldsymbol{\tau} = \operatorname{sgn} t_{i,j+1}^{(j)} \left(t_{i,j+1}^{(j)} \cdot \mathbf{t}_m^{(j)} - t_{m,j+1}^{(j)} \cdot \mathbf{t}_i^{(j)}\right) \tag{4.9}$$

and append them to $\mathbf{F}^{(j+1)}$. When condition (4.4) is passed, condition (4.6) has to be tested and any row $\mathbf{t}_l^{(j+1)}$ failing this check has to be cancelled in $\mathbf{F}^{(j+1)}$. $\mathbf{T}^{(j+1)}$ is obtained by

$$\mathbf{T}^{(j+1)} = \begin{pmatrix} \mathbf{B}^{(j+1)} \\ \mathbf{F}^{(j+1)} \end{pmatrix}. \tag{4.10}$$

3. The previous phase of the algorithm ends with $\mathbf{T}^{(n)}$. Finally,

$$\mathbf{T}^{(n+1)} = \begin{pmatrix} \mathbf{B}^{(n)} \\ -\mathbf{B}^{(n)} \\ \mathbf{F}^{(n)} \end{pmatrix} = \begin{pmatrix} \mathbf{M}_{\text{rev}}^{(n)} \\ -\mathbf{M}_{\text{rev}}^{(n)} \\ \mathbf{M}_{\text{irr}}^{(n)} \end{pmatrix}. \tag{4.11}$$

The reversible and irreversible elementary modes are represented by the rows of $\pm\mathbf{M}_{\text{rev}}^{(n)}$ and $\mathbf{M}_{\text{irr}}^{(n)}$, respectively.

It is worth noting that the steady-state equation (2.2) is equivalent to the equation

$$\mathbf{BNV} = \mathbf{0} \tag{4.12}$$

with $\mathbf{B}$ being any non-singular $n \times n$ matrix. Therefore, the matrix

$$\mathbf{N}' = \mathbf{BN} \tag{4.13}$$

can be used as a transformed stoichiometry matrix in the algorithm. In particular, it is sensible to use that matrix $\mathbf{B}$ that transforms $\mathbf{N}$ into a row-reduced echelon form, which involves ρ linearly independent rows and $n - \rho$ null rows. Application of the algorithm to $\mathbf{N}'$ with its null rows cancelled leads to the same outcome as application to $\mathbf{N}$.

It is often meaningful to gain information about the overall consumption or production of external metabolites in the elementary modes. This can be obtained by formally writing down a stoichiometry matrix for the externals and multiplying it by the vectors $\mathbf{V}^*$ representing elementary modes. This method is easier to perform than including stoichiometric coefficients for the externals in the tableaux and processing them in the algorithm.

5. Justification of the algorithm

The transition from $\mathbf{T}^{(j-1)}$ to $\mathbf{T}^{(j)}$ can be regarded as brought about by premultiplying $\mathbf{T}^{(j)}$ by a matrix, $\mathbf{A}^{(j)}$,

$$\mathbf{T}^{(j)} = \mathbf{A}^{(j)}\mathbf{T}^{(j-1)}. \tag{5.1}$$

So $\mathbf{T}^{(j)}$ is obtained from $\mathbf{T}^{(0)}$ by

$$\mathbf{T}^{(j)} = \mathbf{A}^{(j)}\mathbf{A}^{(j-1)} \ldots \mathbf{A}^{(1)}\mathbf{T}^{(0)}. \tag{5.2}$$

An analogous equation holds for the submatrices $\mathbf{N}^{(j)^{\mathrm{T}}}$ and $\mathbf{M}^{(j)}$, respectively. As in the Gauss-Jordan elimination method (cf. Groetsch and King, 1988), the submatrix $\mathbf{M}^{(j)}$ keeps track of the transformations applied to $\mathbf{N}^{\mathrm{T}}$, that is,

$$\mathbf{N}^{(j)^{\mathrm{T}}} = \mathbf{M}^{(j)}\mathbf{N}^{\mathrm{T}} \tag{5.3}$$

for all $j = 0, 1, \ldots, n$. This can easily be proved by the method of induction with respect to j.

To understand the transitions between the tableaux $\mathbf{T}^{(j)}(j = 0, 1, 2, \ldots, n)$, it is useful to consider hypothetical reaction systems which have the same set of reactions as the system under consideration and in which all metabolites indexed by i with $i = 1, 2, \ldots, j$ are internal whereas all others are external (i.e., their concentrations are considered constant). Let $\Sigma_j (j = 0, 1, 2, \ldots)$ denote these hypothetical systems. In steady states of system Σ_j, we have

$$\sum_{l=1}^{r} n_{il} v_l = 0, \quad i = 1, 2, \ldots, j. \tag{5.4}$$

The flux modes of system Σ_j are defined by condition (C2) and the modified steady-state condition (5.4). This implies that any flux mode of $\Sigma_j (j > 0)$ is also a flux mode of Σ_{j-1}.

Lemma 5. *All row vectors* $\mathbf{m}_i^{(j)}$ *fulfil the steady-state condition (5.4) for system* Σ_j*, i.e.,*

$$\sum_{l=1}^{r} n_{hl} m_{i,l}^{(j)} = 0, \quad h = 1, \ldots, j. \tag{5.5}$$

Proof. We have

$$t_{i,j}^{(j)} = 0 \quad \text{for all } i, \tag{5.6}$$

by construction. This implies that

$$t_{i,h}^{(j)} = 0 \quad \text{for all } i, h \text{ with } 1 \leq h \leq j, \tag{5.7}$$

because any null column again gives a null column in the following tableau. By eqs. (5.3) and (5.7),

$$0 = t_{i,h}^{(j)} = \sum_{l=1}^{r} m_{i,l}^{(j)} n_{hl}, \quad 1 \leq h \leq j, \tag{5.8}$$

which completes the proof.

In particular, Lemma 5 says that the vectors of $\mathbf{M}^{(n+1)}$ being part of the final tableau are all solutions of the steady-state condition $\mathbf{NV} = \mathbf{0}$ for the complete reaction system. The successive tableaux represent a sequence in which the steady-state condition is satisfied for each metabolite one by one. Thus, clearing the jth column of $\mathbf{N}^{(j-1)}$ corresponds to imposing the steady-state requirement on the jth metabolite.

Lemma 5 also has implications for systems involving conservation relations of the form

$$\sum_{i=1}^{n} b_{ki} n_{ij} = 0, \quad k = 1, \ldots, n - \rho \tag{5.9}$$

with the b_{ki} being constant coefficients (for the notion of conservation relations see Reder, 1988; Heinrich and Schuster, 1996). Equation (5.9) implies that if the steady-state equation (5.4) is fulfilled for ρ metabolites corresponding to linearly independent rows in the stoichiometry matrix, then it is also fulfilled for all other metabolites. When going through the columns of the tableaux in our algorithm, therefore, one will find columns which consist of zeroes only. The algorithm could therefore be shortened by a suitable arrangement of columns, so that after only ρ steps, all columns of $\mathbf{N}^{(\rho),\mathrm{T}}$ are null columns. This arrangement, however, requires knowledge of the linear dependencies among the columns, which can be obtained by row reduction of $\mathbf{N}$. If such a reduction is applied, one obtains some null rows in the stoichiometry matrix, which can then be cancelled from the initial tableau (see Section 4).

Lemma 6. *All row vectors of* $\mathbf{B}^{(j)}$ *and* $\mathbf{F}^{(j)}$ *satisfy the sign restriction (C2), in particular*

$$f_{i,n+l}^{(j)} \geq 0 \quad \textit{if reaction } \mathrm{R}_l \textit{ is irreversible,} \tag{5.10}$$

$$b_{i,n+l}^{(j)} = 0 \quad \textit{if reaction } \mathrm{R}_l \textit{ is irreversible.} \tag{5.11}$$

Proof. (by the method of induction). For $j = 0$, inequalities (5.10) and (5.11) are fulfilled since any $f_{i,l}^{(0)}$ equals either 0 or 1, and $b_{i,l}^{(0)} = 0$ for all indices l belonging to irreversible reactions.

Assume that relations (5.10) and (5.11) hold true for $j = k$. For any row $\mathbf{m}_i^{(k+1)}$ originating from a row $\mathbf{m}_h^{(k)}$ with $m_{h,k+1}^{(k)} = 0$, relations (5.10) and (5.11) hold true due to the above assumption. If the row results from the linear combination (4.5), Eq. (5.11) is satisfied due the induction hypothesis, since a linear combination of zeroes gives zero again. If the row results from the linear combination (4.8), then

$$\phi_{n+l} = \left| t_{i,k+1}^{(k)} \right| \cdot t_{m,n+l}^{(k)} + \left| t_{m,k+1}^{(k)} \right| \cdot t_{i,n+l}^{(k)}, \tag{5.12}$$

which is, due to the induction hypothesis, non-negative since reaction R_l is irreversible. If a row $\mathbf{f}_i^{(k+1)}$ results from the linear combination (4.9), we have, due to the induction hypothesis,

$$\tau_{n+l} = \left| t_{i,k+1}^{(k)} \right| \cdot t_{m,n+l}^{(k)} \geq 0 \tag{5.13}$$

for all indices l corresponding to irreversible reactions. This completes the proof.

Theorem 1. *Every reversible elementary mode of* $\Sigma_i (i = 0, \ldots, n)$ *is represented by a row of* $\mathbf{M}_{\mathrm{rev}}^{(i)}$ *or* $-\mathbf{M}_{\mathrm{rev}}^{(i)}$ *and every irreversible elementary mode of* $\Sigma_i (i = 0, \ldots, n)$ *is represented by a row of* $\mathbf{M}_{\mathrm{irr}}^{(i)}$.

Theorem 2. *All rows of the matrices* $\mathbf{M}_{\mathrm{rev}}^{(k)}$, $-\mathbf{M}_{\mathrm{rev}}^{(k)}$ *and* $\mathbf{M}_{\mathrm{irr}}^{(k)}$ *are representatives of different elementary modes to system* Σ_k.

Due to their length, the proofs to Theorems 1 and 2 are given in the Appendices. The two Theorems form a complete justification to the algorithm.

6. Example

To illustrate the applicability of the refined algorithm to biochemical systems, we analyse a reaction scheme representing part of nucleotide metabolism, as shown in Fig. 4. In many cell types such as erythrocytes, adenine and hypoxanthine can be exchanged through the cell membrane. As the concentrations of the external pools, ADext and HYPXext (abbreviations are defined in the legend to Fig. 4), can be considered to be fairly constant in comparison to the varying concentrations inside the cell, these pools are treated as what we call 'external metabolites.' ATP and ADP are considered as externals as well because they participate in many other reactions besides those of nucleotide metabolism. AMP, in contrast, is considered here as an intermediate. Moreover, ribulose 5-phosphate is treated as external because this building block of nucleotides is provided by the pentose phosphate pathway. Reactions AMPDA, NUCI, NUCII, AdPRT, RPPK, HGPRT, ADA, and AK can be treated as irreversible in the direction indicated (cf. Joshi & Palsson,

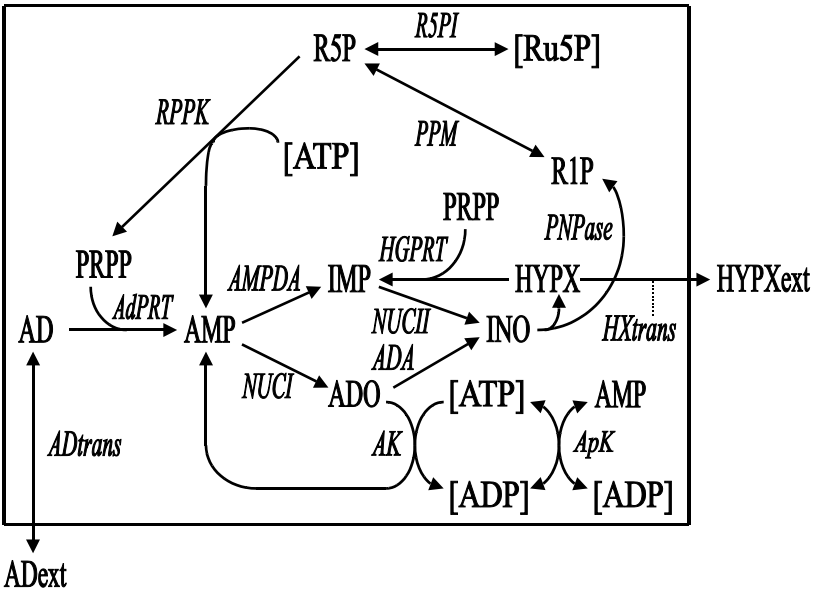

Fig. 4. Scheme of the main reactions of adenine nucleotide metabolism. Reversible reactions (indicated by double arrowheads): *ADtrans*, adenine transport; *ApK*, adenylate kinase; *HXtrans*, hypoxanthine transport; *PNPase*, purine nucleoside phosphorylase; *PPM*, phosphopentomutase; *R5PI*, ribose 5-phosphate epimerase. Irreversible reactions: *ADA*, adenosine deaminase; *AdPRT*, adenine phosphoribosyltransferase; *AK*, adenosine kinase; *AMPDA*, AMP deaminase; *HGPRT*, hypoxanthine phosphoribosyltransferase; *NUCI*, *NUCII*, 5′-nucleotidase dephosphorylating AMP and IMP, respectively; *RPPK*, ribose-phosphate pyrophosphokinase. Abbreviations of metabolites: AD, adenine; ADext, adenine external to the cell; AMP, adenosine monophosphate; ADP and ATP are the corresponding di- and triphosphates; ADO, adenosine; HYPX, hypoxanthine; HYPXext, hypoxanthine external to the cell; IMP, inosine monophosphate; INO, inosine; PRPP, phosphoribosylpyrophosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate.

1989). Note that 5′-nucleotidase (NUC) is a multifunctional enzyme, as it can dephosphorylate all 5′-ribonucleotides. Here, we consider two of these functions, notably the dephosphorylation of AMP and IMP. Since these catalytic functions need not be operative simultaneously, we should treat them formally as two distinct enzymes (denoted here NUCI and NUCII). The stoichiometry matrix then reads

$$\mathbf{N} = \begin{pmatrix} 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & -1 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 0 & -1 & 0 \\ 0 & 0 & 0 & -1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & -1 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & -1 & 0 & -1 & 0 & 1 \end{pmatrix} \begin{matrix} \text{R1P} \\ \text{R5P} \\ \text{PRPP} \\ \text{IMP} \\ \text{INO} \\ \text{AD} \\ \text{ADO} \\ \text{HYPX} \\ \text{AMP} \end{matrix}$$

The columns correspond to reactions in the sequence as given in the legend to Fig. 4. Reactions R1–R6 are reversible, while the remaining reactions are irreversible. This matrix has full row rank. Accordingly, its null-space has dimension five.

Applying the algorithm set out above, we obtain 11 elementary modes, all of which are irreversible:

1: NUCI AK
2: (−2 ApK) ADtrans −R5PI AdPRT RPPK
3: −ApK PNPase PPM NUCII RPPK HGPRT
4: ApK PNPase PPM HXtrans R5PI AMPDA NUCII
5: −ApK PNPase PPM ADtrans HXtrans AMPDA NUCII AdPRT RPPK
6: ApK PNPase PPM HXtrans R5PI NUCI ADA
7: −ApK PNPase PPM ADtrans HXtrans NUCI AdPRT RPPK ADA
8: (2 PNPase) (2 PPM) ADtrans (2 HXtrans) R5PI (2 AMPDA) (2 NUCII)AdPRT RPPK
9: (2 PNPase) (2 PPM) ADtrans (2 HXtrans) R5PI (2 NUCI) AdPRT RPPK (2 ADA)
10: (2 PNPase) (2 PPM) HXtrans R5PI AMPDA (2 NUCII) RPPK HGPRT
11: (2 PNPase) (2 PPM) HXtrans R5PI NUCI NUCII RPPK HGPRT ADA

For example, mode 1 can be understood in that the sum of the 9^{th} and 12^{th} columns (which correspond to the reactions AK and NUCI) of the above stoichiometry matrix is the null vector. The largest number of enzymes used in an elementary mode is nine, in accordance with Lemma 4. Multiplication of the 11 modes with the stoichiometric coefficients of the external species gives the overall stoichiometries:

1: ATP = ADP
2: ADext + 3 ATP + Ru5P = 4 ADP
3: 2 ATP = 2 ADP

4: 2 ADP = HYPXext + ATP + Ru5P
5: ADext + 2 ATP = HYPXext + 2 ADP
6: 2 ADP = HYPXext + ATP + Ru5P
7: ADext + 2 ATP = HYPXext + 2 ADP
8: ADext + ATP = 2 HYPXext + Ru5P
9: ADext + ATP = 2 HYPXext + Ru5P
10: ATP = HYPXext + Ru5P
11: ATP = HYPXext + Ru5P

Modes 1 and 3 are futile cycles. While mode 1 is easy to recognize as such a cycle in the scheme (phosphorylation of adenosine and dephosphorylation of AMP), mode 3 is relatively complex as it encompasses six enzymes.

An important function of human erythrocytes in addition to oxygen transport is the transport of purine bases from organs with a purine surplus to organs in which purines are required (cf. Salerno and Giacomello, 1985). This function is realized by modes 5 and 7, in which adenine is taken up into the cell and hypoxanthine is excreted. Although the uptake and excretion processes do not take place in the same organ, it is sensible to use the concept of elementary modes (which is based on the assumption of steady state) because it can be assumed that the metabolite concentrations averaged over a sufficiently long time span are indeed stationary. Although modes 5 and 7 have the same overall stoichiometry, they slightly differ in their routes. While in mode 5, AMP is deaminated first and dephosphorylated afterwards (by AMPDA and NUCII, respectively), it is the other way round in mode 7 (via NUCI and ADA). Similar functions are performed by modes 8 and 9, but they are accompanied by the degradation of ATP into hypoxanthine and Ru5P.

In mode 2, adenine nucleotides are formed from pentose and adenine. This is an example of a nucleotide salvage pathway (cf. Stryer, 1995). On such a pathway, purine nucleotides can be synthesized from preformed purine bases, which is simpler and much less costly than the *de novo* synthesis from amino acids, tetrahydrofolate and CO_2. In particular, it is of importance in cells that lack *de novo* synthesis of nucleotides, such as erythrocytes (Joshi and Palsson, 1989) and platelets (Holmsen et al., 1979). Modes 4 and 6, in contrast, describe the opposite process of the cleavage of adenine nucleotides into pentose and adenine. Note that neither the route nor the overall stoichiometry of these modes are exactly the opposite of those of the synthesis process (mode 2). While the free energy of the hydrolysis of 3 ATP to 3 ADP is needed to produce one molecule of (a further) ADP, the degradation of one ADP only yields one ATP (from another ADP molecule). Mode 4 and 6 as well as modes 10 and 11 again differ in the usage of AMPDA and NUCII versus NUCI and ADA. In modes 10 and 11, adenine nucleotides are degraded as well. They differ from modes 4 and 6 in that they do not produce ATP in the adenylate kinase reaction but, on the contrary, utilize ATP in the RPPK reaction. The PRPP formed is used to transform part of the hypoxanthine back to IMP, which is then, however, dephosphorylated again. The degradation pathways are important, for example, for the digestion of nucleotides in carnivores.

7. Discussion and computer implementation

The concept of elementary modes. In the Introduction, we outlined the reasons why it is of interest in biotechnology to detect the simplest reaction routes. Here, we have derived several properties of these routes (called elementary flux modes or direct reaction routes). In particular, we have proved that each admissible flux distribution is a non-negative linear combination of elementary modes, as well as a lemma on the minimum number of zero entries (number of enzymes not used) in any one elementary mode. Moreover, we analysed how the reaction routes change when irreversible enzymes are made reversible. These properties are useful in the modelling of biochemical systems because the directionality of a reaction may not be known. Furthermore, we have proved the equivalence of two simplicity conditions used alternatively in the definition of elementary modes.

We have here presented in detail an algorithm for detecting all elementary flux modes for (bio)chemical reaction systems of any complexity and have proved its efficiency. As will be discussed below, this algorithm combines various routines presented earlier by other authors for computing related invariants of reaction systems. We felt that it was worth giving, in the biomathematical literature, a sound and self-contained proof of this algorithm. The main difference from most of the other algorithms is that reversible reactions need not be decomposed into irreversible steps. We do not claim to have found the fastest algorithm for the case when both reversible and irreversible reactions are present, all the more since running time is not the only criterion: memory requirement is important as well due to the possible occurrence of a large number of elementary modes. It is certainly worth improving the algorithm in the future to cope with larger biochemical networks on the scale of whole cell metabolism.

Comparison with alternative algorithms. Seressiotis and Bailey (1988) and Mavrovouniotis *et al.* (1990) developed methods for constructing, by computer, simple routes in biochemical networks leading from some given substrate(s) to some given product(s). A distinction was made between required reactants, allowed reactants (which may or may not be consumed by the pathway), intermediates, required products and allowed (by)products, while we only make a distinction between internal and external metabolites. Their algorithms are similar to ours in that routes are constructed in a step-wise manner by imposing the steady-state condition consecutively on the intermediates, under consideration of sign restrictions. Their handling of reversible reactions is, however, complicated because they divide each of these into two opposite irreversible steps, which then have to be checked at each step of the algorithm to ensure they do not occur in the same route. (This drawback was later removed by Mavrovouniotis, 1992). Furthermore, they recognized the problem of redundant routes, but did not give test criteria phrased in a clear mathematical way. In our algorithm, the conditions (4.4) and (4.6) serve to exclude occurrence of redundant modes (i.e., non-elementary modes and duplicate elementary modes) in the tableaux.

Several authors have proposed using the theory of Petri nets for modelling biochemical networks (Hofestädt, 1994; Reddy *et al*., 1996). Petri nets are graphs with two different types of vertices: places and transitions (cf. Reisig, 1985; Starke,

1990), which, as for biochemical networks, correspond to metabolites and reactions, respectively. Interestingly, in Petri net theory, there is a counterpart to elementary modes: the minimal T-invariants (cf. Starke, 1990). They are defined by condition (C3′) rather than (C3). However, transitions in Petri nets are usually defined as unidirectional (irreversible). Bidirectional processes have to be split into forward and reverse transitions, with the drawback outlined above.

Colom and Silva (1990) gave a detailed overview of several algorithms for computing the minimal T-invariants. They are based on row operations in tableaux as well. (A simplified algorithm lacking a test condition such as relation (4.4) and, hence, yielding not only minimal T-invariants, is given in Starke (1990)). Basically, Colom and Silva (1990) propose two alternative tests for eliminating non-minimal invariants, which are related to our test conditions (4.4) and (4.6). However, as in the algorithm proposed by Nožička *et al*. (1974), condition (4.4) is phrased in such a way that, on the right-hand side, $\mathbf{m}_l^{(j)}$ rather than $\mathbf{m}_l^{(j+1)}$ is considered; that is, the comparison is made with all rows in the old tableau rather than with those already situated in the new tableau. If all reactions are irreversible, this is a suitable condition. If, however, some reactions are reversible, it can be shown that the condition is sometimes too strict because three rows can exist which prevent each other from entering the new tableau after linear combination of two of these. Therefore, we modified this condition by writing $\mathbf{m}_l^{(j+1)}$. This, however, sometimes allows non-elementary rows to enter the new tableau because, when the test is made, not enough rows are present, against which the test can be performed. To eliminate them, we have included condition (4.6), which is related to the alternative proposal of Colom and Silva (1990). These authors suggested to test each row after computation of a new tableau (or only after computation of the last tableau), to see whether the row's support (i.e., the set of transition vertices included) contains, as a proper subset, the support of some other row situated in the new tableau. If so, it is discarded. If such a test were included, condition (4.4) would not be necessary. However, to save memory occupation, it is favourable to eliminate as many non-minimal modes as early as possible because of the fast (often exponential) increase in the number of edges of a cone as the dimension of the problem increases. On the other hand, one should find a compromise between memory requirement and execution time. According to our experience, criterion (4.6) is rarely violated. To save computational time, it is therefore sufficient to apply it only upon computing the final tableau.

Schilling *et al*. (2000) presented an algorithm for computing 'extreme pathways.' These essentially correspond to the vectors of what is called the convex basis in Pfeiffer *et al*. (1999), and their algorithm is similar to the procedure for computing the convex basis given in Nožička *et al*. (1974) and briefly sketched in Pfeiffer *et al*. (1999). The special feature of the approach by Schilling *et al*. (2000) is that external metabolites are treated formally as internal and exchange reactions are included that connect them with external pools. All reversible reactions that are not exchange reactions are decomposed into irreversible forward and reverse steps. However, this leads to an unnecessary increase in the number of reactions and requires the above-mentioned elimination of a number of cyclic modes. The convex basis has the advantage that the number of the vectors involved is usually

smaller than the number of elementary modes. On the other hand, it has the drawback that biochemically important pathways such as glycolysis are not obtained as extreme pathways when reversible side reactions are included in the system (see Introduction and Fig. 1). Moreover, it may change considerably upon addition of enzymes to the system.

The concept of elementary modes is related to the direct mechanisms in chemistry, which give the simplest routes of reaction steps combined in specific proportions such that the net conversion involves only initial reactants and final products. We suggest that the algorithm presented here can be used for computing direct mechanisms. Mavrovouniotis (1992) presented an algorithm for computing all direct mechanisms for any reaction system consisting of reversible and irreversible reactions. It includes a routine to eliminate duplicate and non-direct reaction mechanisms. After computation of a tableau, all mechanisms are classified according to their length. Mechanisms of length 1 are certainly direct. From among the mechanisms of length 2, all those are discarded that involve a reaction representing a length-one mechanism (cf. Lemma 2). Next, all mechanisms of length 3 are checked whether they involve one of the length-one or length-two mechanisms and so on. This test is related to the second condition proposed by Colom and Silva (1990) but is more elaborate because it involves a classification of modes according to their length. It is difficult to compare the running times of Mavrovouniotis' algorithm and ours. Condition (4.4) has a time requirement that is quadratic in the size of the tableau (for a given pair of rows, an intersection set has to be computed and tested against all other rows). Condition (4.6) and related tests (e.g. the test routine proposed by Mavrovouniotis, 1992) roughly needs a time linear in tableau size. (Note that the intersection set on the r.h.s. of relation (4.6) is known already when condition (4.4) has been passed, and it need not be determined for all pairs i, m but belongs to a given pair under consideration.) However, Mavrovouniotis' algorithm requires computation of mechanisms (perhaps with large numbers, which causes computational problems) that are eliminated afterwards. Our condition (4.4) allows us to avoid computation of redundant mechanisms to a certain extent. A limited number of such modes can still arise, but they are eliminated by condition (4.6). Our method has the advantage that conditions (4.4) and (4.6) are used during computation of the new tableau, while Mavrovouniotis (1992) proposed using his condition only after the new tableau has been completely calculated.

An earlier algorithm presented by Happel and Sellers (1982, 1989), which was later translated into graph-theoretical concepts (Zeigarnik and Temkin, 1994) initially assumes that all steps are reversible and finally discards those mechanisms that use an irreversible reaction in the wrong direction. This again entails an unnecessary, substantial memory requirement. The method starts from the null-space matrix $\mathbf{K}$, which can be computed very fast. In the algorithm, the observation is used that at least $\gamma - 1$ components of any vector representing a direct mechanism are zero, with γ being the dimension of the null-space (proved here in Lemma 4). Thus, $\gamma - 1$ rows are chosen from $\mathbf{K}$. Now, the columns of the submatrix formed by these $\gamma - 1$ rows are linearly combined so as to give a null matrix. The coefficients of this combination are calculated by Kramer's rule. Applying the same linear combination to the columns of the entire matrix $\mathbf{K}$ gives a vector representing a

direct mechanism, provided that all irreversible steps are used in the right direction. The algorithm requires an exhaustive search of all $\binom{r}{\gamma-1}$ possibilities of choosing $\gamma - 1$ rows from **K**. If γ is relatively small or not very different from r, this algorithm is reasonably fast. When, however, γ is of the order of $r/2$ and r is large, the number of possibilities to be tested is so large that the algorithm certainly requires more time than the algorithm presented here.

Futile cycles. Our definition of elementary modes allows cyclic modes, and deliberately so, because biochemical reactions are often bimolecular with the second reactant or product being a cofactor such as ATP or NADH, which are often considered external (i.e. their concentrations are considered constant). This is also an important difference between the methods of Seressiotis and Bailey (1988) and Mavrovouniotis *et al.* (1990) and our algorithm. The former provide cyclic modes only if the source and sink metabolites driving these cycles energetically (e.g. ATP and ADP) are specified as substrate and product. Our algorithm automatically yields all cycles without the need for making the externals explicit. The analysis of cyclic modes can help us understand the physiological relevance of futile cycles so frequent in metabolism (cf. Baranyai and Blum, 1989; Fell, 1993). An example is provided by the fructose-2,6-bisphosphate cycle (cf. Hers and Hue, 1983). One can calculate the energy cost attributable to substrate cycling. Note that the principle of detailed balancing (cf. Walz and Caplan, 1988) cannot be applied to cycles when external metabolites are "hidden" in reaction cycles. In contrast, cyclic modes that are not driven by energy-rich compounds cannot operate on their own, due to the principle of detailed balancing.

Computer implementation. The algorithm given in Section 4 has been implemented in the computer programs METATOOL (Pfeiffer *et al*., 1999) written in C (compiled with GNU gcc and portable to many platforms) and EMPATH, which has been written by one of the authors (J.H.W.) in VisualWorks\Smalltalk (Park Place Systems, Sunnyvale). Both programs are freely available from ftp://mudshark.brookes.ac.uk/pub/software/ibmpc for academic users, METATOOL also from http://www.bioinf.mdc-berlin.de/projects/metabolic/. They use reaction equations as input. These programs are able to cope with reaction networks that are so complex that several thousands of modes occur. The programs include a routine for dividing a newly generated row by the greatest common denominator of its elements, giving what Colom and Silva (1990) call a canonical flow. In the above presentation, we have not dealt with this issue because it is straightforward to implement in computer programs.

A tool for interpreting metabolism. We have demonstrated the usefulness of the presented algorithm by an example from nucleotide metabolism. Most of the detected routes are so complex that they can hardly be found by inspection. Other examples given earlier (Schuster *et al*., 1996, 2000; Liao *et al*., 1996) also show that systems of even moderate size usually give rise to quite complex elementary modes. Our algorithm provides a tool for finding these routes in an automated way. Several of the elementary modes correspond to well-known pathways, such as the salvage pathway in the example considered here and glycolysis in a system considered earlier (Schuster *et al*., 2000) while some others correspond to routes

that are not described in biochemical textbooks. For these, it has to be checked for each particular cell type under study whether the regulatory structure allows them to function.

Acknowledgements. We would like to thank Thomas Pfeiffer (formerly Berlin, now Zurich), Klaus Mauch (Stuttgart) and Dr. Peter Starke (Berlin) for stimulating discussions. We are grateful to Dr. Monike Heiner (Cottbus) for drawing our attention to the minimal T-invariants.

8. Appendix 1: Proof of Theorem 1

Since the system Σ_0 has no internal metabolites, any vector with two or more non-zero components does not fulfil condition (C3) for system Σ_0, since it can be decomposed into vectors with only one non-zero component each. Any vector with one non-zero component is a multiple of some row of the identity matrix contained in $\mathbf{M}^{(0)}$, which is duly partitioned into two parts corresponding to reversible and irreversible reactions.

Assume now that the theorem holds true for Σ_j. Suppose there is an elementary mode, $\mathbf{V}^*$, of Σ_{j+1} that is not represented by one of the rows mentioned in Theorem 1 (with $i = j + 1$). Since $\mathbf{V}^*$ is also a flux mode of Σ_j, we can distinguish the following cases.

Case a) $\mathbf{V}^*$ is an elementary mode of Σ_j. It then represents, due to the induction hypothesis, the same mode as some row $\mathbf{m}_k^{(j)}$. Since $\mathbf{V}^*$ is a flux mode of Σ_{j+1}, it fulfils the steady-state condition

$$\sum_{l=1}^{r} n_{j+1,l} v_l^* = 0, \tag{A.1}$$

which implies

$$\sum_{l=1}^{r} n_{j+1,l} m_{k,l}^{(j)} = 0. \tag{A.2}$$

By Eq. (5.3), we conclude

$$t_{k,j+1}^{(j)} = 0. \tag{A.3}$$

So $\mathbf{t}_k^{(j)}$ goes into tableau $\mathbf{T}^{(j+1)}$ in the algorithm. It stays there even upon application of condition (4.6) because if it failed that condition, then the mode obtained by linear combination of $\mathbf{m}_l^{(j)}$ and $\mathbf{m}_m^{(j)}$ would also be a mode of system Σ_j. This would be a contradiction of Lemma 2 and $\mathbf{m}_k^{(j)}$ being an elementary mode of Σ_j. Accordingly, $\mathbf{V}^*$ is represented by one of the rows mentioned in Theorem 2 (with $i = j + 1$).

If $\mathbf{V}^*$ is no elementary mode of Σ_j, it can, due to Lemma 1, be decomposed into such modes, which are, due to the induction hypothesis, involved in $\mathbf{M}^{(j)}$,

$$\mathbf{V}^* = \sum_k \eta_k \mathbf{m}_k^{(j)}, \ \eta_k \in \mathrm{R}, \tag{A.4}$$

$$\mathrm{S}(\mathbf{V}^*) \subset \mathrm{S}\left(\mathbf{m}_k^{(j)}\right). \tag{A.5}$$

Case b) Exactly two coefficients η_k, say $\eta_{k'}$ and $\eta_{k''}$, are unequal to zero. From the steady-state condition (A.1), we conclude that

$$\eta_{k'} \sum_{l=1}^{r} n_{j+1,l} m_{k',l}^{(j)} + \eta_{k''} \sum_{l=1}^{r} n_{j+1,l} m_{k'',l}^{(j)} = 0. \tag{A.6}$$

By Eq. (5.3), this gives

$$\eta_{k'} t_{k',j+1}^{(j)} + \eta_{k''} t_{k'',j+1}^{(j)} = 0. \tag{A.7}$$

Since $\eta_{k'}, \eta_{k''} \neq 0$, Eq. (A.7) implies that if $t_{k',j+1}^{(j)} = 0$, then also $t_{k'',j+1}^{(j)} = 0$. In this case, both $\mathbf{m}_{k'}^{(j)}$ and $\mathbf{m}_{k''}^{(j)}$ would represent flux modes of Σ_{j+1}. However, the decomposition (A.4) would then be a contradiction of the assumption that $\mathbf{V}^*$ is an elementary mode of Σ_{j+1}. The remaining possibility to consider is that both $t_{k',j+1}^{(j)}$ and $t_{k'',j+1}^{(j)}$ are unequal to zero. We can then distinguish three subcases:

Case b1) Both $\boldsymbol{t}_{k'}^{(j)}$ and $\boldsymbol{t}_{k''}^{(j)}$ belong to $\mathbf{F}^{(j)}$. On account of Lemma 2, there is at least one index l with $m_{k',l}^{(j)} = 0$ and $m_{k'',l}^{(j)} \neq 0$ and one index h with $m_{k',h}^{(j)} \neq 0$ and $m_{k'',h}^{(j)} = 0$. Thus, $\eta_{k'}$ and $\eta_{k''}$ have to be positive, because otherwise $\mathbf{V}^*$ would violate the sign restrictions for l and h in Σ_j. So $t_{k',j+1}^{(j)}$ and $t_{k'',j+1}^{(j)}$ have to have opposite signs. As $\mathbf{V}^*$ and any positive multiples of $\mathbf{V}^*$ are representatives of the same flux mode (see Definition 1), we can put

$$\eta_{k'} = \left| t_{k'',j+1}^{(j)} \right|. \tag{A.8}$$

Equations (A.7) and (A.8) imply

$$\eta_{k''} = \left| t_{k',j+1}^{(j)} \right|. \tag{A.9}$$

Inspection of Eq. (4.8) shows that $\phi = \mathbf{V}^*$ with $i = k', m = k''$. If $\boldsymbol{t}_{k'}^{(j)}$ and $\boldsymbol{t}_{k''}^{(j)}$ fulfil condition (4.4) and their combination fulfils condition (4.6), then $\mathbf{V}^*$ representing an elementary mode to Σ_{j+1} is contained in the tableau $\mathbf{M}_{\mathrm{irr}}^{(j)}$. If conditions (4.4) or (4.6) are not fulfilled, there is a vector $\mathbf{m}_{l\cdot}^{(j+1)}$ belonging to $\mathbf{M}_{\mathrm{irr}}^{(j)}$ with

$$\mathrm{S}\left(\mathbf{m}_{k'}^{(j)}\right) \cap \mathrm{S}\left(\mathbf{m}_{k''}^{(j)}\right) \subseteq \mathrm{S}\left(\boldsymbol{m}_l^{(j+1)}\right). \tag{A.10}$$

This would imply $\mathrm{S}(\mathbf{V}^*) \subseteq \mathrm{S}(\boldsymbol{m}_l^{(j+1)})$. Lemma 2 as applied to system Σ_{j+1} would then imply that $\mathbf{V}^*$ and $\boldsymbol{m}_l^{(j+1)}$ represent the same elementary mode, so that this mode is included in the tableau.

Case b2) Both $\boldsymbol{t}_{k'}^{(j)}$ and $\boldsymbol{t}_{k''}^{(j)}$ belong to $\mathbf{B}^{(j)}$. Due to the scaling indeterminacy property, we can choose

$$\eta_{k'} = t_{k'',j+1}^{(j)}. \tag{A.11}$$

So we obtain, from Eq. (A.7),

$$\eta_{k''} = -t^{(j)}_{k',j+1}. \tag{A.12}$$

Inspection of Eq. (4.5) reveals that either $\mathbf{V}^* = \boldsymbol{\theta}$ or $\mathbf{V}^* = -\boldsymbol{\theta}$ with $i = k', m = k''$. If $\boldsymbol{t}^{(j)}_{k'}$ and $\boldsymbol{t}^{(j)}_{k''}$ fulfil condition (4.4) and their combination fulfils condition (4.6), then either $\mathbf{V}^*$ or $-\mathbf{V}^*$ (both representing a reversible elementary mode to Σ_{j+1}) is contained in the tableau $\mathbf{M}^{(j+1)}_{\text{rev}}$. If conditions (4.4) or (4.6) are not fulfilled, there is a vector $\boldsymbol{m}^{(j+1)}_l$ belonging to $\mathbf{M}^{(j+1)}_{\text{rev}}$ which has zero elements wherever both $\mathbf{m}^{(j)}_{k'}$ and $\mathbf{m}^{(j)}_{k''}$ have. So it has zero elements wherever $\mathbf{V}^*$ has, a contradiction of Lemma 2.

Case b3) $\boldsymbol{t}^{(j)}_{k'}$ and $\boldsymbol{t}^{(j)}_{k''}$ belong to $\mathbf{F}^{(j)}$ and $\mathbf{B}^{(j)}$, respectively. (The opposite case can be treated analogously.) For all indices l corresponding to irreversible reactions, $m^{(j)}_{k'',l} = 0$. So we have $\eta_{k''} > 0$ because if not, $\mathbf{V}^*$ would violate the sign restriction for Σ_j. Consequently, we can choose $\eta_{k'}$ and $\eta_{k''}$ so that $\mathbf{V}^* = \boldsymbol{\tau}$ as calculated in Eq. (4.9) with $i = k', m = k''$. Thus, if $\mathbf{m}^{(j)}_{k'}$ and $\mathbf{m}^{(j)}_{k''}$ fulfil condition (4.4) and their combination fulfils condition (4.6), then $\mathbf{V}^*$ is contained in the tableau $\mathbf{M}^{(j+1)}_{\text{irr}}$. If conditions (4.4) or (4.6) are not fulfilled, a similar argument as in case b1) applies.

Case c) At least three coefficients η_k in Eq. (A.4) (say $\eta_{k'}$, $\eta_{k''}$, and $\eta_{k'''}$) are non-zero. Then $t^{(j)}_{k,j+1} \neq 0$ for $k = k', k''$, and k''', since if not, one of the rows $\mathbf{m}^{(j)}_k$ would represent a flux mode of Σ_{j+1}, so that the decomposition (A.4), (A.5) would be a contradiction of the assumption that $\mathbf{V}^*$ is an elementary mode of Σ_{j+1}. Therefore the set $\{k', k'', k'''\}$ contains a pair of indices, say (k', k''), for which

$$\operatorname{sgn}\left(m^{(j)}_{k',j+1}\right) = -\operatorname{sgn}\left(m^{(j)}_{k'',j+1}\right) \neq 0. \tag{A.13}$$

Now consider the vector

$$\mathbf{V}^{**} = \operatorname{sgn}\left(t^{(j)}_{k',j+1}\right)\left(t^{(j)}_{k',j+1} \cdot \mathbf{t}^{(j)}_{k''} - t^{(j)}_{k'',j+1} \cdot \mathbf{t}^{(j)}_{k'}\right). \tag{A.14}$$

If both $\boldsymbol{t}^{(j)}_{k'}$ and $\boldsymbol{t}^{(j)}_{k''}$ are rows of $\mathbf{F}^{(j)}$, then $\mathbf{V}^{**}$ is an admissible flux vector for Σ_{j+1}, since $\mathbf{V}^{**}$ equals $\boldsymbol{\phi}$ as calculated in Eq. (4.8), due to relation (A.13). If both $t^{(j)}_{k'}$ and $\boldsymbol{t}^{(j)}_{k''}$ are rows of $\mathbf{B}^{(j)}$, then $\mathbf{V}^{**}$ and $-\mathbf{V}^{**}$ are admissible flux vectors for Σ_{j+1}, since they equal $\boldsymbol{\theta}$ and $-\boldsymbol{\theta}$ as calculated in Eq. (4.5). If one out of those two vectors (say $\mathbf{t}^{(j)}_{k'}$) belongs to $\mathbf{B}^{(j)}$ and the other one belongs to $\mathbf{F}^{(j)}$, $\mathbf{V}^{**}$ is again an admissible flux vector for Σ_{j+1}, since $\mathbf{V}^{**}$ equals $\boldsymbol{\tau}$ as calculated in Eq. (4.9). Clearly, $\mathrm{S}(\mathbf{m}^{(j)}_{k'}) \cap \mathrm{S}(\mathbf{m}^{(j)}_{k''}) \subseteq \mathrm{S}(\mathbf{V}^{**})$. So we can conclude, by consideration of relation (A.5), that $\mathrm{S}(\mathbf{V}^*) \subseteq \mathrm{S}(\mathbf{V}^{**})$. By Lemma 2, $\mathbf{V}^*$ and $\mathbf{V}^{**}$ are representatives of the same elementary mode. So case b) applies, since $\mathbf{V}^*$ can be decomposed into $\mathbf{m}^{(j)}_{k'}$ and $\mathbf{m}^{(j)}_{k''}$. As we have considered all possible cases, the proof is complete.

9. Appendix 2: Proof of Theorem 2

Due to Lemmata 5 and 6, the rows of $\mathbf{M}^{(k)}$ fulfil conditions (C1) and (C2) for system Σ_k. So only condition (C3) remains to be considered. We use the method of induction with respect to the index k. The system Σ_0 has no internal metabolites, so that the rows of the identity matrix represent elementary modes. For modes containing reversible reactions only, also the negative of these rows represent elementary modes.

Assume now that Theorem 2 holds true for $k = j$. Consider first those rows of $\mathbf{T}^{(j)}$ for which $t^{(j)}_{i,j+1} = 0$. By Lemma 5, we then have

$$\sum_{l=1}^{r} m^{(j)}_{i,l} n_{k+1,l} = 0. \tag{A.15}$$

Due to this equation and Lemma 6, $\mathbf{m}^{(j)}_i$ represents a flux mode of system Σ_{j+1}. It is, moreover, an elementary mode of this system since otherwise it could be decomposed into two flux modes which then also were flux modes of Σ_j so that $\mathbf{m}^{(j)}_i$ could not represent an elementary mode of Σ_j.

Now we prove that the vector $\boldsymbol{\theta}$ obtained from Eq. (4.5) represents an elementary mode to Σ_{j+1} if the rows $\mathbf{m}^{(j)}_i$ and $\mathbf{m}^{(j)}_m$ entering Eq. (4.5) fulfil condition (4.4) and the vector $\boldsymbol{\theta}$ taken as $\mathbf{m}^{(j+1)}_l$ satisfies condition (4.6) for any pair of rows $\mathbf{m}^{(j)}_i$, $\mathbf{m}^{(j)}_m$ tested subsequently. If it were not, it could be decomposed into at least two elementary modes of Σ_{j+1} as in Eq. (3.1) with $\mathrm{S}(\mathbf{m}_l) \supset \mathrm{S}(\boldsymbol{\theta})$. Due to Theorem 1, for any of these elementary modes, either $\mathbf{m}_l$ or $-\mathbf{m}_l$ would be involved in $\mathbf{M}^{(j+1)}$. In particular, they would belong to $\mathbf{M}^{(j+1)}_{\mathrm{rev}}$, since a reversible flux mode can be decomposed into reversible flux modes only (cf. Lemma 1). Now a contradiction arises, because in the algorithm, none of these rows $\mathbf{m}_l$ can enter the tableau $\mathbf{T}^{(j+1)}$ before $\boldsymbol{\theta}$, because otherwise, $\boldsymbol{\theta}$ would not fulfil condition (4.4). Moreover, they cannot enter the tableau $\mathbf{T}^{(j+1)}$ after $\boldsymbol{\theta}$ either, because otherwise, $\boldsymbol{\theta}$ would be eliminated due to condition (4.6), which provides a sort of competition between combinations of "old rows" and the rows already in the new tableau.

Similarly, one can prove that the vectors $\boldsymbol{\phi}$ and $\boldsymbol{\tau}$ obtained from Eqs. (4.8) and (4.9) are elementary. If they were not, they could be expressed into vectors $\mathbf{m}_l$ with at least one of them involved in $\mathbf{M}^{(j+1)}_{\mathrm{irr}}$. So conditions (4.4) or (4.6) would be violated for $\mathbf{m}_l$, $\boldsymbol{\phi}$ or $\boldsymbol{\tau}$.

If two or more rows mentioned in Theorem 2 represented the same mode, they would have identical sets $\mathrm{S}(\boldsymbol{m}^{(k)}_i)$. This is, however, impossible because, in the algorithm, condition (4.4) is applied, which ensures no mode goes from $\mathbf{T}^{(k-1)}$ into $\mathbf{T}^{(k)}$ twice. Therefore, all rows represent different elementary modes, as stated in Theorem 2. As we have considered all possible cases, the proof is complete.

References

Baranyai, J.M., Blum, J.J.: Quantitative analysis of intermediary metabolism in rat hepatocytes incubated in the presence and absence of ethanol with a substrate mixture including ketoleucine. *Biochem. J.* **258**, 121–140 (1989)

Benevolensky, S.V., Clifton, D., Fraenkel, D.G.: The effect of increased phosphoglucose isomerase on glucose metabolism in *Saccharomyces cerevisiae. J. Biol. Chem* . **269**, 4878–4882 (1994)

Chernikov, S.N.: *Linear Inequalities* (in Russian), Moscow: Nauka, 1968

Clarke, B.L.: Complete set of steady states for the general stoichiometric dynamical system. *J. Chem. Phys.* **75**, 4970–4979 (1981)

Colom, J.M., Silva, M.: Convex geometry and semiflows in P/T nets. A comparative study of algorithms for computation of minimal P-semiflows, in Advances in Petri Nets 1990, G. Rozenberg (Ed.), Lecture Notes in Computer Science 483. Berlin: Springer, 79–112 (1990)

Dandekar, T., Schuster, S., Snel, B., Huynen, M., Bork, P.: Pathway alignment: Application to the comparative analysis of glycolytic enzymes. *Biochem. J.* **343**, 115–124 (1999)

Fell, D.A.: Substrate cycles: theoretical aspects of their role in metabolism. *Comm. theor. Biol.* **6**, 1–14 (1990)

Fell, D.A.: The analysis of flux in substrate cycles, in Modern Trends in Biothermokinetics, S. Schuster, M. Rigoulet, R. Ouhabi and J.-P. Mazat (Eds), New York: Plenum, 97–101 (1993)

Fell, D.A., Small, J.R.: Fat synthesis in adipose tissue: an examination of stoichiometric constraints. *Biochem. J.* **238**, 781–786 (1986)

Fishtik, I., Datta, R.: A thermodynamic approach to the systematic elucidation of unique reaction routes in catalytic reactions. *Chem. Eng. Sci* . **55**, 4029–4043 (2000)

Groetsch, C.W., King, J.T.: *Matrix methods and applications*, Englewood Cliffs: Prentice Hall, 1988

Happel, J., Sellers, P.H.: Multiple reaction mechanisms in catalysis. *Ind. Eng. Chem. Fundam*. **21**, 67–76 (1982)

Happel, J., Sellers, P.H.: The characterization of complex systems of chemical reactions. *Chem. Eng. Commun.* **83**, 221–240 (1989)

Heinrich, R., Schuster, S.: *The Regulation of Cellular Systems*, New York: Chapman & Hall, 1996

Hers, H.G., Hue, L.: Gluconeogenesis and related aspects of glycolysis. *Ann. Rev. Biochem.* **52**, 617–653 (1983)

Hofestädt, R.: A petri net application to model metabolic processes. *Syst. Anal. Mod. Simul.* **16**, 113–122 (1994)

Holmsen, H., Smith, J.B., Daniel, J.L., Holme, S., Bills, T.K.: Platelet biochemistry, in *Hematology*, **1**, R.M. Schmidt (Ed.) Boca Raton: CRC Press, 273–303 (1979)

Horiuti, J., Nakamura, T.: Stoichiometric number and the theory of steady reaction. *Z. Phys. Chem., Neue Folge* **11**, 358–365 (1957)

Joshi, A., Palsson, B.O.: Metabolic dynamics in the human red cell. I. A comprehensive kinetic model. *J. theor. Biol.* **141**, 515–528 (1989)

Karp, P.D., Krummenacker, M., Paley, S., Wagg, J.: Integrated pathway-genome databases and their role in drug discovery. *Trends Biotechnol.* **17**, 275–281 (1999)

Klamt, S., Schuster, S., Gilles, E.D.: Calculability analysis in underdetermined metabolic networks illustrated by a model of the central metabolism in purple nonsulfur bacteria. *Biotechnol. Bioeng*. **77**, 734–751 (2002)

Leiser, J., Blum, J.J.: On the analysis of substrate cycles in large metabolic systems. *Cell Biophys*. **11**, 123–138 (1987)

Liao, J.C., Hou, S.-Y., Chao, Y.-P.: Pathway analysis, engineering and physiological considerations for redirecting central metabolism. *Biotechnol. Bioeng*. **52**, 129–140 (1996)

Mavrovouniotis, M.L.: Synthesis of reaction mechanisms consisting of reversible and irreversible steps. 2. Formalization and analysis of the synthesis algorithm. *Ind. Eng. Chem. Res.* **31**, 1637–1653 (1992)

Mavrovouniotis, M.L., Stephanopoulos, G., Stephanopoulos. G.: Computer-aided synthesis of biochemical pathways. *Biotechn. Bioeng.* **36**, 1119–1132 (1990)

Milner, P.C.: The possible mechanisms of complex reactions involving consecutive steps. *J. Electrochem. Soc.* **111**, 228–232 (1964)

Nožička, F., Guddat, J., Hollatz, H., Bank, B.: *Theorie der linearen parametrischen Optimierung*, Berlin: Akademie-Verlag, 1974

Nuño, J.C., Sánchez-Valdenebro, I., Pérez-Iratxeta, C., Meléndez-Hevia, E., Montero, F.: Network organization of cell metabolism: monosaccharide interconversion. *Biochem. J.* **324**, 103–111 (1997)

Pfeiffer, T., Sánchez-Valdenebro, I., Nuño, J.C., Montero, F., Schuster, S.: METATOOL: For studying metabolic networks, *Bioinformatics* **15**, 251–257 (1999)

Reddy, V.N., Liebmann, M.N., Mavrovouniotis, M.L.: Qualitative analysis of biochemical reaction systems. *Comp. Biol. Med.* **26**, 9–24 (1996)

Reder, C.: Metabolic Control Theory: A structural approach. *J. theor. Biol.* **135**, 175–201 (1988)

Reisig, W.: *Petri Nets: An Introduction*, Berlin: Springer, 1985

Rockafellar, R.T.: *Convex Analysis*, Princeton (NJ): Princeton University Press, 1970

Salerno, C., Giacomello, A.: Hypoxanthine-guanine exchange by intact human erythrocytes. *Biochemistry* **24**, 1306–1309 (1985)

Schilling, C.H., Schuster, S., Palsson, B.O., Heinrich, R.: Metabolic pathway analysis: Basic concepts and scientific applications in the post-genomic era. *Biotechnol. Prog*. **15**, 296–303 (1999)

Schilling, C.H., Letscher, D., Palsson, B.O.: Theory for the systemic definition of metabolic pathways and their use in interpreting metabolic function from a pathway-oriented perspective. *J. theor. Biol.* **203**, 229–248 (2000)

Schilling, C.H., Palsson, B.O.: Assessment of the metabolic capabilities of *Haemophilus influenzae* Rd through a genome-scale pathway analysis. *J. theor. Biol.* **203**, 249–283 (2000)

Schuster, S., Höfer, T.: Determining all extreme semi-positive conservation relations in chemical reaction systems: A test criterion for conservativity. *J. Chem. Soc. Faraday Trans.* **87**, 2561–2566 (1991)

Schuster, S., Schuster, R.: Detecting strictly detailed balanced subnetworks in open chemical reaction networks. *J. Math. Chem.* **6**, 17–40 (1991)

Schuster, R., Schuster, S.: Refined algorithm and computer program for calculating all non-negative fluxes admissible in steady states of biochemical reaction systems with or without some flux rates fixed. *Comp. Appl. Biosci*. **9**, 79–85 (1993)

Schuster, S., Hilgetag, C.: On elementary flux modes in biochemical reaction systems at steady state. *J. Biol. Syst.* **2**, 165–182 (1994)

Schuster, S., Hilgetag, C., Woods, J.H., Fell, D.A.: Elementary modes of functioning in biochemical networks, in *Computation in Cellular and Molecular Biological Systems*, R. Cuthbertson, M. Holcombe and R. Paton (Eds), Singapore: World Scientific, 151–165 (1996)

Schuster, S., Fell, D.A., Dandekar, T.: A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. *Nature Biotechnol*. **18**, 326–332 (2000)

Selkov, E., Maltsev, N., Olsen, G.J., Overbeek, R., Whitman, W.B.: A reconstruction of the metabolism of *Methanococcus jannaschii* from sequence data. *Gene* **197**, GC11–GC26 (1997)

Sellers, P.H.: Combinatorial classification of chemical mechanisms. *SIAM J. Appl. Math.* **44**, 784–792 (1984)

Seressiotis, A., Bailey, J.E.: MPS: An artificially intelligent software system for the analysis and synthesis of metabolic pathways. *Biotechn. Bioeng.* **31**, 587–602 (1988)

Starke, P.H.: *Analyse von Petri-Netz-Modellen.* Stuttgart: Teubner, 1990

Stephanopoulos, G.N., Aristidou, A.A., Nielsen, J.: *Metabolic Engineering*. San Diego: Academic Press, 1998

Stryer, L.: *Biochemistry*, New York: Freeman, 1995

Walz, D., Caplan, S.R.: Energy coupling and thermokinetic balancing in enzyme kinetics. *Cell Biophys.* **12**, 13–28 (1988)

Zeigarnik, A.V., Temkin, O.N.: A graph-theoretical model of complex reaction mechanisms: Bipartite graphs and the stoichiometry of complex reactions. *Kinet. Catal.* **35**, 647–655 (1994)