



The Role of Stoichiometric Analysis in Studies of Metabolism: An Example

ATHEL CORNISH-BOWDEN*[†] AND JAN-HENDRIK S. HOFMEYR[‡]

**Bioénergétique et Ingénierie des Protéines, Institut Fédératif “Biologie Structurale et Microbiologie”, Centre National de la Recherche Scientifique, 31 Chemin Joseph-Aiguier, B.P. 71, 13402 Marseille Cedex 20, France* and [‡]*Department of Biochemistry, University of Stellenbosch, Stellenbosch 7600, South Africa*

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Stoichiometric analysis uses matrix algebra to deduce the constraints implicit in metabolic networks. When applied to simple networks, it can often give the impression of being an unnecessarily complicated way of arriving at information that is obvious from inspection, for example, that the sum of the concentrations of the adenine nucleotides is constant. Applied to a more complicated example, that of glycolysis in *Trypanosoma brucei*, it yields information that is far from obvious and may have importance for developing therapeutic ways of eliminating this parasite. Even in simplified form, the network contains nine reactions or transport steps involving 11 metabolites. This immediately shows that there must be at least two stoichiometric constraints, and indeed two can be recognized by inspection: conservation of adenine nucleotides and conservation of the two forms of NAD. There is, however, a third, which involves eight different phosphorylated intermediates in non-obvious combinations and is very difficult to recognize by inspection. It is also difficult to recognize by inspection that no fourth stoichiometric constraint exists. Gaussian elimination provides a systematic way of analysing a network in such a way that all the stoichiometric relationships that it contains emerge automatically.

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Introduction

Reder (1988) emphasized the need to recognize the invariants in biochemical systems and to separate properties that depend on the kinetic properties of components from that depend only on stoichiometric structure. In this way, she introduced stoichiometric analysis to metabolic control analysis (Kacser *et al.*, 1995) and prepared the way for powerful methods of pathway analysis (e.g. Schuster *et al.*, 2000) that are becoming essential tools for making sense of the mountains of data generated by genome

sequencing (Cornish-Bowden & Cárdenas, 2000), as well as for more general applications in biotechnology (e.g. Liao, 2000; Rohwer & Hofmeyr, 2000; Rohwer & Botha, 2001). Nonetheless, metabolic control analysis is sometimes said to be excessively concerned with algebra (e.g. Gutfreund, 1991), more so, indeed, than with understanding metabolic regulation. Even some people with some sympathy with the broad ideas of metabolic control analysis come close to this opinion when they see the effort that goes into explaining how stoichiometric analysis is done. Is it really worthwhile to go through all the linear algebra to arrive at a conclusion that may appear obvious from inspection without any

[†] Author to whom correspondence should be addressed.
E-mails: athel@ibsm.cnrs-mrs.fr; jhsh@maties.sun.ac.za

algebra (for example, that the sum of the concentrations of reduced and oxidized NAD is conserved)? Even if it is not obvious, what does it matter? Why should we care if a particular combination of concentrations is conserved?

To try to make all this clear, we shall examine stoichiometric analysis in the context of a particular example that illustrates both that the conservation constraints are not always obvious and that they can have practical consequences, for example in choosing a strategy for combating a major disease.

Glycolysis in *Trypanosoma brucei*

The form of the parasitic protozoan *Trypanosoma brucei* that infects human blood and causes African sleeping sickness has a relatively simple metabolism. It depends wholly on its host for a supply of glucose, oxygen and other nutrients, and leaves regulation of their availability entirely to the host (Opperdoes, 1987; Michels *et al.*, 2000). Moreover, despite its aerobic metabolism, it has no tricarboxylate cycle but simply excretes pyruvate into the host bloodstream. It is thus heavily dependent on glycolysis, and has one of the highest known glycolytic activities for a eukaryote. It carries out glycolysis and a few other reactions in a special organelle called the *glycosome*, and for the purposes of this discussion, we can consider its energy metabolism to follow the scheme shown in Fig. 1, a simplified version of the scheme developed into a computer model by Bakker *et al.* (1997, 1999). This metabolism is normally aerobic, but it can proceed anaerobically, and so we can ignore the mitochondrial conversion of glycerol 3-phosphate into dihydroxyacetone phosphate, and we shall also initially ignore the transport of these two metabolites between the glycosome and the cytosol.

Even this simplified scheme is too complicated to be analysed on paper as it stands (though it is easily within the scope of computer programs in current use for metabolic modelling), but further simplifications result from treating glucose 6-phosphate and fructose 6-phosphate as one species, and from drawing the boundaries of the system at the boundaries of the glycosome,

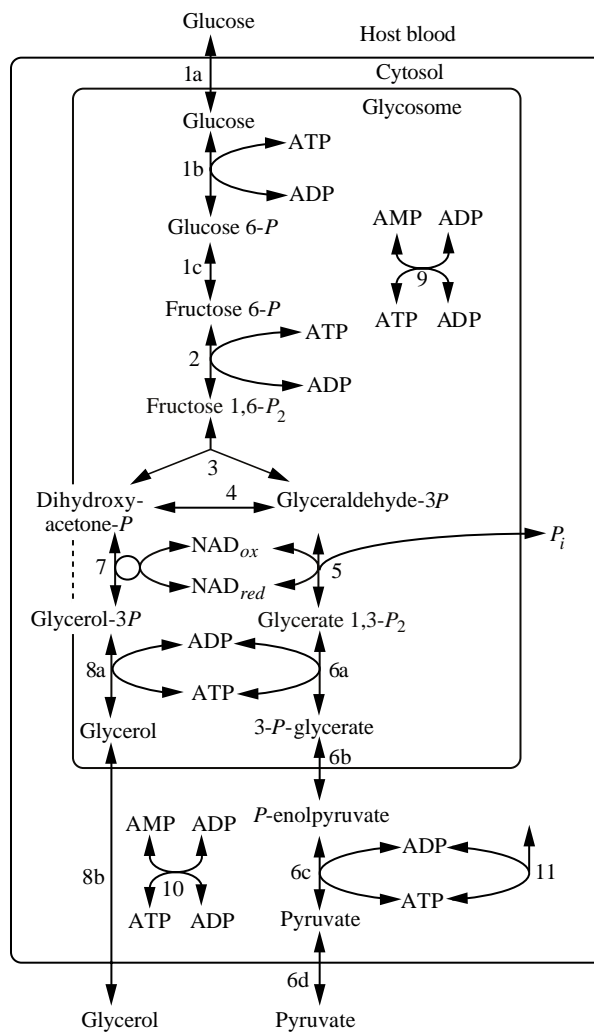


FIG. 1. Anaerobic glycolysis in *Trypanosoma brucei*. The aerobic process is divided between four compartments, of which three (host blood, cytosol and glycosome) are shown; the fourth, the mitochondrion, is inactive in anaerobic glycolysis. The boundary of the glycosome is shown by a broken line in the vicinity of dihydroxyacetone phosphate and glycerol 3-phosphate because these metabolites are partitioned between the glycosome and the cytosol. The enzyme-catalysed reactions and transport steps are numbered as follows: 1a, glucose transport; 1b, hexokinase; 1c, hexose phosphate isomerase; 2, phosphofructokinase; 3, aldolase; 4, triose phosphate isomerase; 5, glyceraldehyde 3-phosphate dehydrogenase; 6a, phosphoglycerate kinase; 6b, enolase, pyruvate kinase and phosphoenolpyruvate transport (treated as a single process); 6c, pyruvate kinase; 6d, pyruvate transport; 7, glycerol 3-phosphate dehydrogenase; 8a, glycerol kinase; 8b, glycerol transport; 9, adenylate kinase in the glycosome; 10, adenylate kinase in the cytosol; 11, "growth" (all ATP-consuming processes other than those explicitly shown).

effectively treating glucose, glycerol, pyruvate and inorganic phosphate as fixed metabolites rather than as intermediates (Fig. 2). This

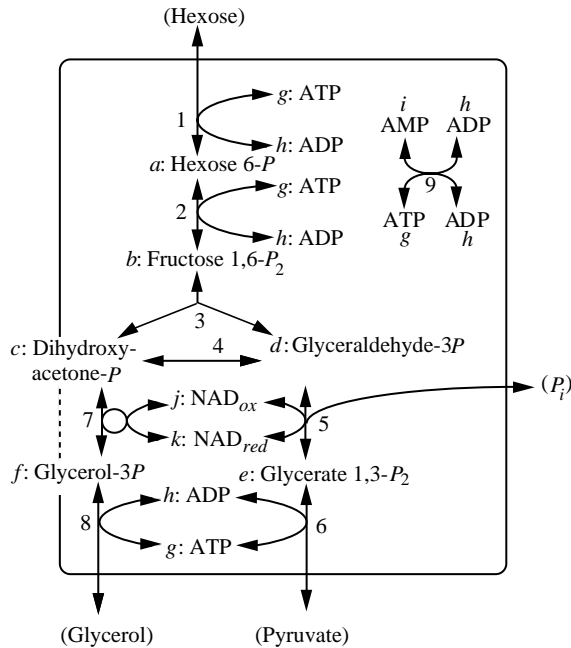


FIG. 2. Simplified form of the scheme shown in Fig. 1, reactions 10 and 11 being omitted and the distinction between glycosome and cytosol ignored. Metabolites that are treated as fixed reservoirs in the analysis (hexose, glycerol, pyruvate and inorganic phosphate) are shown in parentheses. The following short symbols for the intermediates are defined: *a*, hexose 6-phosphate; *b*, fructose 1,6-bisphosphate; *c*, dihydroxyacetone phosphate; *d*, glyceraldehyde 3-phosphate; *e*, 1,3-bisphosphoglycerate; *f*, glycerol 3-phosphate; *g*, ATP; *h*, ADP; *i*, AMP; *j*, NAD_{ox} ; *k*, NAD_{red} . These correspond to those used in the text in equations and for labelling the columns of the augmented stoichiometric matrix that refer to the rates of change of the metabolite concentrations. Likewise, the numbers 1–9 that label the nine processes are also used in the text to label the columns of the stoichiometric matrix, and defined in the legend to Fig. 1, with the simplification that reaction 1 combines reactions 1a–1c of Fig. 1, reaction 6 combines reactions 6a–6d, and reaction 8 combines reactions 8a and 8b.

is unsatisfactory for modelling trypanosomal metabolism, but it is adequate for arriving at a first approximation of the stoichiometric constraints.

For the algebraic development we shall use the metabolite symbols shown in Fig. 2: *a*, hexose 6-phosphate; *b*, fructose 1,6-bisphosphate; *c*, dihydroxyacetone phosphate; *d*, glyceraldehyde 3-phosphate; *e*, 1,3-bisphosphoglycerate; *f*, glycerol 3-phosphate; *g*, ATP; *h*, ADP; *i*, AMP; *j*, NAD_{ox} ; *k*, NAD_{red} . The rate at which any metabolite concentration changes can then be expressed as the sum of the rates of the reactions

that produce it minus the sum of the rates of those that consume it (multiplied by any appropriate stoichiometric coefficients). For hexose 6-phosphate, for example, the rate of change of its concentration is da/dt , the difference between the rates of reactions 1 and 2:

$$\frac{da}{dt} = v_1 - v_2 \quad (1)$$

and the other metabolites may be handled similarly:

$$\frac{db}{dt} = v_2 - v_3, \quad (2)$$

$$\frac{dc}{dt} = v_3 - v_4 - v_7, \quad (3)$$

$$\frac{dd}{dt} = v_3 + v_4 - v_5, \quad (4)$$

$$\frac{de}{dt} = v_5 - v_6, \quad (5)$$

$$\frac{df}{dt} = v_7 - v_8, \quad (6)$$

$$\frac{dg}{dt} = -v_1 - v_2 + v_6 + v_8 + v_9, \quad (7)$$

$$\frac{dh}{dt} = v_1 + v_2 - v_6 - v_8 - 2v_9, \quad (8)$$

$$\frac{di}{dt} = v_9, \quad (9)$$

$$\frac{dj}{dt} = -v_5 + v_7, \quad (10)$$

$$\frac{dk}{dt} = v_5 - v_7. \quad (11)$$

Some of these equations are more complicated than others because some metabolites are involved in more processes than others. For example, eqn (7), referring to ATP, has five terms on the right-hand side because ATP

appears in Fig. 2 in five different reactions. Although in nearly all cases the stoichiometric coefficients are -1 , 0 or 1 , a coefficient of 2 appears in eqn (8) because two molecules of ADP are produced by reaction 9. Careful comparison between this set of 11 equations and the diagram in Fig. 2 will confirm that both contain all of the same stoichiometric information about the 11 intermediate metabolites and the nine reactions.

To analyse this information, it is convenient to assemble the equations into a single *stoichiometric matrix*:

$$\begin{matrix} & \begin{matrix} 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 \end{matrix} \\ \begin{bmatrix} 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & -1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & -1 & 0 & 0 & -1 & 0 & 0 \\ 0 & 0 & 1 & 1 & -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 \\ -1 & -1 & 0 & 0 & 0 & 1 & 0 & 1 & 1 \\ 1 & 1 & 0 & 0 & 0 & -1 & 0 & -1 & -2 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & -1 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & -1 & 0 & 0 \end{bmatrix} & \end{matrix} \quad (12)$$

Columns of this matrix represent steps in the pathway, for example, column 3 represents aldolase. These meanings remain unchanged during the algebraic development, in contrast to the meanings of the rows, which, in the initial state before any matrix operations, correspond to metabolite concentrations $a-k$. For example, row 1 initially contains exactly the same information as eqn (1) and expresses the rate of change of a . The value of 1 in column 1 means that the metabolite in question is a product of reaction 1, the value of -1 in column 2 that is a substrate of reaction 2, and the values of 0 in all the other columns that it is not a substrate or a product of any of the other reactions. In the same way, row 2 corresponds to eqn (2) and expresses the rate of change of b , and so on.

However, just as we could add eqn (1) to eqn (2) to produce a new relationship that would no longer refer uniquely to the metabolite concentration a , so also rows of the stoichiometric matrix can be combined to give new rows, but once this is done it ceases to be possible to associate each row with a unique metabolite. For this reason, the rows are not labelled as a, b , etc. or in any other way that might suggest fixed meanings. To make it possible to retrace all the row operations, therefore, the stoichiometric matrix is *augmented* with the elements of a unit matrix of order 11:

$$\begin{matrix} & \begin{matrix} 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 \end{matrix} & & \begin{matrix} a & b & c & d & e & f & g & h & i & j & k \end{matrix} \\ \begin{bmatrix} 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & \vdots & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & \vdots & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & -1 & 0 & 0 & -1 & 0 & 0 & \vdots & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 1 & -1 & 0 & 0 & 0 & 0 & \vdots & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & \vdots & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & \vdots & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ -1 & -1 & 0 & 0 & 0 & 1 & 0 & 1 & 1 & \vdots & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 & 0 & -1 & 0 & -1 & -2 & \vdots & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & \vdots & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & -1 & 0 & 1 & 0 & 0 & \vdots & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & -1 & 0 & 0 & \vdots & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix} & \end{matrix} \quad (13)$$

The extra columns are labelled with symbols for the metabolites, because their meanings, like those of the first nine columns, remain fixed. The element in each row of a metabolite column then shows how the metabolite is involved in the row in question. For example, row 2 initially contains 1 in column b and 0 in every other column from a to k , hence row 2 of the stoichiometric matrix refers initially to the rate of change of b . All the columns of the augmented matrix will be subjected to the same row operations, and the elements in columns a – k will provide a systematic record of these operations.

Before proceeding further, consider the following question: how many stoichiometric constraints between the metabolite concentrations does the scheme in Fig. 2 imply, and how many of them are obvious from inspecting it? By a stoichiometric constraint we mean a conservation relationship of the kind that applies to the reduced and oxidized forms of NAD: as the scheme makes no provision for net synthesis or consumption of NAD, the sum of the concentrations of the two forms must be constant. There are 11 rows but only 9 columns in the original matrix (12), so it is immediately clear that there must be at least two conservation relationships. Moreover, two such relationships are obvious from inspection, and it takes very little time to guess that $[ATP] + [ADP] + [AMP]$ and $[NAD_{ox}] + [NAD_{red}]$ are conserved, and not much more time to confirm that they are indeed conserved. If there were any doubts about it, then one could see immediately that the entries in the groups of rows corresponding to these metabolites add up to zero in every column, implying that the sum of their rates of concentration change is zero and, therefore, the sum of their concentrations is constant.

If this were all there were to it, one might indeed conclude that even a scheme as complicated as the one in Fig. 2 could be analysed by inspection and the algebra left to those who like algebra. There is, however, a third relationship, and even if one happened to notice it, could one be sure that there was not a fourth? Readers who notice the third relationship immediately and can tell by inspection that there is not a fourth can ignore the algebra, but otherwise the analysis is necessary.

Stoichiometric Analysis

First recall what any given row in the matrix means in terms of metabolite flows in the steady state. For example, consider row 8, initially representing ADP (we choose this row as it has the largest number of different values in it, but any row will do). As ADP is a variable metabolite, the rate of change of its concentration in the steady state must be zero (from the definition of a steady state), but now 8 defines this rate (under any conditions, not just in the steady state) as follows:

$$\frac{dh}{dt} = v_1 + v_2 - v_6 - v_8 - 2v_9 \quad (14)$$

and similarly (arbitrarily taking row 2, for fructose 1,6-bisphosphate), we have

$$\frac{db}{dt} = v_2 - v_3. \quad (15)$$

In the steady state, both of these rates are equal to zero, but the relationships as written apply in all circumstances, not just in the steady state. Any linear combination at all of the two expressions still produces a valid relationship. Thus, for example, by adding row 8 for ADP to -2 times row 2 for fructose 1,6-bisphosphate, we can obtain a new row 8 that defines a different relationship:

$$\frac{dh}{dt} - 2\frac{db}{dt} = v_1 - v_2 + 2v_3 - v_6 - v_8 - 2v_9. \quad (16)$$

However, although this illustrates the general principle that any row in a matrix can be replaced by a linear combination of it with other rows without affecting the validity of the information contained in the matrix, it is not very helpful because it is unlikely that haphazard linear combinations of the rows of a large matrix will ever lead to a useful piece of information. What is needed is a systematic way of proceeding that will guarantee the extraction of all the useful information that the matrix contains; this is what Gaussian elimination (Strang, 1993) provides.

Gaussian Elimination

Elimination was originally conceived as a procedure for solving simultaneous equations.

It begins by subtracting combinations of the first equation from each of the other equations in such a way that the first unknown is eliminated from all but the first equation; next the second equation is used in the same way to eliminate the second unknown from all the equations apart from the first two. Proceeding in this way through a complete set of equations eventually leads, if the equations have a solution, to a triangular arrangement in which the last equation contains just one unknown, the next-to-last contains just two, and so on. This is very convenient, because the last equation is then trivial to solve, substituting its solution into the next-to-last makes that equation trivial, and so on.

However, although this is how elimination originated, it has a more general utility, because it also extracts the available information from the sets of equations that have no solution (because the equations are inconsistent) and from the sets that have infinitely many (because there are more unknowns than equations). Even if there are equally many unknowns and equations there may not be a unique solution, because the equations may be inconsistent, or because some of the equations may just repeat

information contained in the others. All of this may be recognizable at a glance if one is dealing with a trivial textbook example of two or three equations in two or three unknowns, but it is quite another matter if one has 11 equations in nine unknowns, for example. All that is then obvious is that if the equations are consistent, there must be at least two redundant equations, i.e. ones that are linear combinations of others. However, there is no certainty that there are *only* two redundant equations, and, in fact, in our example there are three.

In this more general case, the reduced matrix may not be triangular, but must be in *row-echelon form*. This means that as far as possible it has non-zero elements on the main diagonal, with only zero elements below; however, when it is impossible to have a non-zero element, the “diagonal” is permitted to shift to the right, as exemplified by matrix (21) below.

The top row in the solution must contain a non-zero element in column 1, and only zero entries below it (eliminating v_1 from all the rows but the first). As rows 7 and 8 contain non-zero entries in column 1, we must eliminate these by adding row 1 to row 7, and subtracting row 1 from row 8:

$$\begin{array}{cccccccccc|cccccccc}
 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & & a & b & c & d & e & f & g & h & i & j & k \\
 \left[\begin{array}{cccccccccc}
 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 1 & -1 & 0 & 0 & -1 & 0 & 0 & 0 \\
 0 & 0 & 1 & 1 & -1 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 \\
 0 & -2 & 0 & 0 & 0 & 1 & 0 & 1 & 1 & 0 \\
 0 & 2 & 0 & 0 & 0 & -1 & 0 & -1 & -2 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
 0 & 0 & 0 & 0 & -1 & 0 & 1 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 1 & 0 & -1 & 0 & 0 & 0
 \end{array} \right] & \begin{array}{l} \vdots \\ \vdots \\ \vdots \\ \vdots \\ \vdots \\ \vdots \\ \vdots \\ \vdots \\ \vdots \\ \vdots \\ \vdots \end{array} & \begin{array}{cccccccccccc}
 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
 1 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
 -1 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1
 \end{array} \right] \quad (17)
 \end{array}$$

All the columns are transformed in the same way, and the change to column *a* provides a

next four columns without any complications, leading to

$$\begin{array}{c}
 \begin{array}{cccccccccc}
 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & \vdots \\
 \left[\begin{array}{cccccccccc}
 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & \vdots \\
 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & \vdots \\
 0 & 0 & 1 & -1 & 0 & 0 & -1 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 2 & -1 & 0 & 1 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & -1 & 1 & 1 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & -2 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & \vdots \\
 0 & 0 & 0 & 0 & 0 & -1 & 1 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & \vdots
 \end{array} \right.
 \end{array}
 \end{array}
 \begin{array}{c}
 \begin{array}{cccccccccccc}
 a & b & c & d & e & f & g & h & i & j & k \\
 \left[\begin{array}{cccccccccccc}
 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
 1 & 2 & 1 & 1 & 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
 -1 & -2 & -1 & -1 & -1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\
 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\
 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 1
 \end{array} \right.
 \end{array}
 \end{array}
 \end{array}
 \tag{18}$$

systematic record of this first transformation: the 1 in row 7 (together with the 1 already in column *g*) shows that this row is now the sum of the original rows 1 and 7, not the original row 7, and the -1 in row 8 similarly records the operation on that row.

At this stage, there is a different kind of problem to resolve because row 6 contains zero in the diagonal. The difficulty can be resolved by exchanging it with row 10 (a transformation equivalent to writing the corresponding equations in a different order, which has no effect on the information they contain):

Eliminating zeroes under the diagonal entries then proceeds in exactly the same way for the

$$\begin{array}{c}
 \begin{array}{cccccccccc}
 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & \vdots \\
 \left[\begin{array}{cccccccccc}
 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & \vdots \\
 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & \vdots \\
 0 & 0 & 1 & -1 & 0 & 0 & -1 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 2 & -1 & 0 & 1 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 0 & -1 & 1 & 0 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & -1 & 1 & 1 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & -2 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & \vdots \\
 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & \vdots
 \end{array} \right.
 \end{array}
 \end{array}
 \begin{array}{c}
 \begin{array}{cccccccccccc}
 a & b & c & d & e & f & g & h & i & j & k \\
 \left[\begin{array}{cccccccccccc}
 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\
 1 & 2 & 1 & 1 & 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\
 -1 & -2 & -1 & -1 & -1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 1 & 0
 \end{array} \right.
 \end{array}
 \end{array}
 \end{array}
 \tag{19}$$

The new row 6 must now be subtracted from now 11 in order to remove the entry of 1 in column 6 of that row, but the procedure is the same as before until column 8, where there is no row exchange that can produce a non-zero diagonal element:

a linear constraint in addition to the two known to exist from the fact that there are two more rows than columns in the unaugmented stoichiometric matrix (12).

1	2	3	4	5	6	7	8	9	⋮	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>	<i>i</i>	<i>j</i>	<i>k</i>
1	-1	0	0	0	0	0	0	0	⋮	1	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	0	0
0	0	1	-1	0	0	-1	0	0	⋮	0	0	1	0	0	0	0	0	0	0	0
0	0	0	2	-1	0	1	0	0	⋮	0	0	-1	1	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	0	0	0	0	-1	1	0	0	⋮	0	0	0	0	1	0	0	0	0	1	0
0	0	0	0	0	0	-1	1	1	⋮	1	2	1	1	1	0	1	0	0	0	0
0	0	0	0	0	0	0	0	-1	⋮	0	0	0	0	0	0	1	1	0	0	0
0	0	0	0	0	0	0	0	1	⋮	0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	1	⋮	1	2	1	1	1	1	1	0	0	0	0
0	0	0	0	0	0	0	0	0	⋮	0	0	0	0	0	0	0	0	0	1	1

(20)

When this happens, there is no choice but to accept a zero and proceed to the next column. The meaning of this diagonal zero is that there is

The next, and last column is then processed as those before, and the final appearance of the matrix as follows:

1	2	3	4	5	6	7	8	9	⋮	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>	<i>i</i>	<i>j</i>	<i>k</i>
1	-1	0	0	0	0	0	0	0	⋮	1	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	0	0
0	0	1	-1	0	0	-1	0	0	⋮	0	0	1	0	0	0	0	0	0	0	0
0	0	0	2	-1	0	1	0	0	⋮	0	0	-1	1	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	0	0	0	0	-1	1	0	0	⋮	0	0	0	0	1	0	0				

Notice that the staircase arrangement of non-zero diagonal elements shifts to the right in row 8, illustrating the difference between a matrix in row-echelon form and a triangular matrix.

The bottom three rows represent conservation constraints, and to interpret these it is helpful to recall the meaning of the top row:

$$\frac{da}{dt} = v_1 - v_2 \quad (22)$$

and to remember that it applies in all conditions, and not just in the steady state. This is still the same as eqn (1), because no row operations have been applied to row 1. However, row 11 no longer represents dk/dt , because of the row operations that have been done, but $(dj/dt) + (dk/dt)$ (as shown by the non-zero metabolite columns in row 11 of the matrix), and so

$$\frac{dj}{dt} + \frac{dk}{dt} = 0. \quad (23)$$

The zero comes from the row of zeroes in the left-hand part of the matrix; it is *not* a consequence of assuming a steady state. As this sum does not depend on the kinetic activity of any enzyme in the system it is a relationship derived from the stoichiometry of the pathway and not from its kinetic properties. As it is only possible for this sum of rates of concentration changes to be zero in all conditions if the sum of the corresponding concentrations is constant, it follows that this row provides the first conservation relationship:

$$[\text{NAD}_{ox}] + [\text{NAD}_{red}] = \text{constant}. \quad (24)$$

The next follows in the same way from row 9:

$$[\text{ATP}] + [\text{ADP}] + [\text{AMP}] = \text{constant}. \quad (25)$$

These are, of course, the two constraints that are obvious from the inspection of the pathway in Fig. 2. The third is not at all obvious, and follows from row 10, which defines the following relationship between rates of concentration changes:

$$\begin{aligned} \frac{da}{dt} + 2\frac{db}{dt} + \frac{dc}{dt} + \frac{dd}{dt} + \frac{de}{dt} + \frac{df}{dt} \\ + 2\frac{dg}{dt} + \frac{dh}{dt} = 0. \end{aligned} \quad (26)$$

When converted into the corresponding relationship between metabolite concentrations, this provides the third stoichiometric constraint:

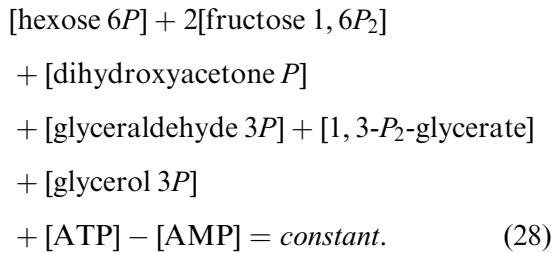
$$\begin{aligned} & [\text{hexose } 6P] + 2[\text{fructose } 1, 6P_2] \\ & + [\text{dihydroxyacetone } P] \\ & + [\text{glyceraldehyde } 3P] \\ & + [1, 3-P_2\text{-glycerate}] + [\text{glycerol } 3P] \\ & + 2[\text{ATP}] + [\text{ADP}] = \text{constant}. \end{aligned} \quad (27)$$

Even once the relationship is pointed out, it is not immediately clear what is being conserved. It is tempting to suggest that transferable phospho groups are conserved, but note that although 1,3-bisphosphoglycerate contains two such groups, it has a coefficient of 1 in the equation, whereas fructose 1,6-bisphosphate and ATP have coefficients of 2. Moreover, even if the original system had been defined to include 3-phosphoglycerate as an intermediate, it would not have appeared in this equation. In fact, the relationship does not involve all transferable phospho groups, but only those that are not accounted for by influx of inorganic phosphate and efflux of 3-phosphoglycerate. In other words, the sum of concentrations shown in eqn (27) cannot change because all the inorganic phosphate that enters the glycosome is exactly balanced by the export of 3-phosphoglycerate (implicit in reaction 6 of Fig. 2, and explicit as reaction 6b of Fig. 1).

The 1-phospho group in 1,3-bisphosphoglycerate derived from inorganic phosphate is not the same one that becomes the 3-phospho group of 3-phosphoglycerate, but this has no importance because there is an exact balance between the two. The fact that they involve different atoms would only matter in a labelling experiment, for example with ^{32}P .

Any linear combination of the conservation equations is itself a valid conservation equation, and Gaussian elimination does not guarantee that the relationships that emerge from the analysis will be the simplest or biochemically most "natural". For example, instead of eqns (25) and (27) one of them might have been replaced by the difference between the two if the initial equations had been written

in a different order:



In this case, the negative coefficient of the AMP concentration makes it more difficult to rationalize what is being conserved, and for purposes of discussion it is usually preferable to find ways of writing the conservation equations that avoid negative coefficients. Schuster and colleagues (Schuster & Höfer, 1991; Schuster & Hilgetag, 1995) have described a technique that guarantees positive coefficients in the conservation equations.

For the sake of simplicity, we have considered only the anaerobic case of trypanosomal metabolism. In the absence of mitochondrial reoxidation of glycerol 3-phosphate, the NAD redox balance can only be maintained by having identical fluxes in the glycerol and pyruvate limbs. In the steady state, therefore, reaction 4, catalysed by triose phosphate isomerase, has a rate of exactly zero in the system as defined, though it can have a non-zero transient rate. If one were interested only in the steady state, one could have omitted reaction 4 from the metabolic scheme altogether, and if this had been done, a fourth conservation relationship would have emerged from the analysis. Note, however, that in the bloodstream trypanosomal metabolism is aerobic, and in this case the steady-state rate through triose phosphate isomerase is not zero, and hence this extra constraint does not apply. A more obvious reaction with zero rate in the steady state is reaction 9, the reaction of adenylate kinase: if this were omitted from the network eqn (25) would also disappear, but the analysis would otherwise be unaffected. In the general case reactions with zero steady-state rates can be recognized as noted by Schuster & Schuster (1991).

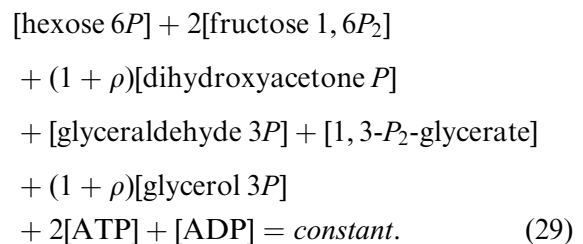
Any ATPase activity in the glycosome would abolish the constraint embodied by eqn (27). It is necessary, therefore, to apply the results of stoichiometric analysis with caution, and to take reasonable care to represent the network struc-

ture as accurately as possible. In reality, there is no evidence for ATPase activity in the glycosome (where it would fulfil no obvious biological function), but there is certainly such activity in the cytosol, and in the full model, as shown in Fig. 2, reaction 11 represents all of the growth processes driven by consumption of ATP.

Effect of Compartmentation

How would the above analysis be altered by including the mitochondrial oxidation of glycerol 3-phosphate and the two additional steps for transport of dihydroxyacetone phosphate and glycerol 3-phosphate between the glycosome and the cytosol? As no net loss of phospho groups results from including these steps, the essential conclusion that a subset of transferable phospho groups is conserved remains unchanged in the full analysis, though now the cytosolic molecules of the two metabolites concerned are included in the conservation equation.

In a single compartment at constant volume, the concentration of every metabolite is proportional to its mass, and so one can loosely speak of the conservation of concentration even though it is really mass that is conserved. However, when there are several compartments with different volumes, it is essential to be more precise, because equal concentrations of a metabolite on the two sides of a membrane do not imply equal masses or vice versa. It should be obvious that what is conserved in the present case is not the total concentration of the phospho groups concerned, but the total mass, and so continued expression of the relation in terms of concentrations must allow for the fact that the cytosol has a volume about 20 times larger than that of the glycosome. Representing the cytosol/glycosome volume ratio as ρ , therefore, the conservation relationship, still in terms of glycosomal concentrations, is now as follows:



It is interesting to ask as to why the glycosome exists at all in *T. brucei* and related parasites. An attractive hypothesis was that concentrating the glycolytic enzymes in a small volume was necessary for the very high glycolytic flux that exists in the parasite (Aman *et al.*, 1985; Misset *et al.*, 1986). However, recent modelling studies (Bakker *et al.*, 2000a) indicate that essentially the same flux would result from the known catalytic activities of the enzymes if they were diluted in the cytosol; on the other hand, the glycosome does appear essential to prevent the accumulation of hexose phosphates to very high concentrations (around 100 mM) that would certainly result in osmotic shock if they occurred in the living organism. The existence of the glycosome thus parallels the better known regulatory mechanisms such as cooperativity and feedback inhibition that occur in many organisms (though not, apparently, in *T. brucei*), whose fundamental role is not so much to control fluxes (which can be controlled quite adequately without them) but to allow fluxes to vary without concomitant large variations in metabolite concentrations (Hofmeyr & Cornish-Bowden, 1991; Cornish-Bowden *et al.*, 1995; Hofmeyr & Cornish-Bowden, 2000; Cornish-Bowden & Cárdenas, 2001). Moreover, this interpretation of the function of the glycosome indicates that the conservation relationship expressed by eqn (29) is more than a haphazard consequence of the network structure that may have some value for biotechnology but is a property of the organism selected during evolution to protect it from osmotic stress.

This example illustrates the importance of modelling for studying metabolic systems of more than a trivial size. Once the model of glycolysis in *T. brucei* had been developed and validated (Bakker *et al.*, 1997), it was easy to examine the effect of modelling the same system in the absence of the glycosome (Bakker *et al.*, 2000a); the corresponding experiment, to study the properties of a living parasite that lacked the glycosome, would be impossible at the present state of knowledge.

Discussion

The above example illustrates how stoichiometric analysis can provide a way of deducing

constraints that are not obvious from inspection of a metabolic pathway. However, it does not explain the utility of the resulting knowledge. What is the practical value of knowing that trypanosomal glycolysis conserves certain phospho groups?

The obvious answer is that this is a necessary piece of information for computer modelling of the pathway. However, although older modelling programs such as MetaModel (Cornish-Bowden & Hofmeyr, 1991) require the user to define the conservation constraints, more recently updated ones such as SCAMP (Sauro, 1993), GEPASI (Mendes, 1993, 1997) and JARNAC (Sauro, 2000) calculate these automatically, and thus, in a sense, absolve the user from any necessity to know about them. However, knowledge of stoichiometric constraints is useful even away from a modelling context, as we now show.

A classic example is provided by anaplerotic pathways. The tricarboxylate cycle is regarded in textbooks (e.g. Lehninger, 1975) as fulfilling two functions, first as a way of coupling pyruvate produced by glycolysis to the aerobic production of ATP, second as a pool of starting materials for biosynthetic pathways. It was not initially realized that it is impossible for the complete cycle to fulfil both functions: as written there is a finite pool of intermediates, none of which can be removed as biosynthetic precursors without depleting the supply of intermediates needed for energy metabolism. Only with the recognition of the stoichiometric constraint did it become evident that short-circuits, such as that provided by the glyoxylate cycle (Krebs & Kornberg, 1957), must exist to allow the pool size to be increased to compensate for any mass removed by biosynthetic pathways.

Metabolism in *T. brucei* provides a different kind of argument. The principal reason for studying this organism at all is the search for ways of eliminating it from infected patients, which can be regarded as the search for ways of modifying its metabolism to the point where it dies. There are two fundamental approaches to this. The more obvious one is to introduce inhibitors that decrease the glycolytic flux to a level that cannot support life. We shall not discuss this approach here (though see Bakker *et al.*, 2000b), apart from noting that it is made

more difficult than it may appear at first sight by the low flux control coefficients of most enzymes.

The second approach is to perturb a metabolite concentration so severely that major toxic effects result. The widely used herbicide glyphosate (or Roundup) does this very efficiently (Boocock & Coggins, 1983), and uncompetitive inhibition of any enzyme can produce very large changes in substrate concentrations if the substrate concentrations in question are unconstrained (Cornish-Bowden, 1986). Almost any of the enzymes in Fig. 1 might seem a suitable candidate for attack by an uncompetitive inhibitor. However, the constraints that we have derived (which can be confirmed and supplemented by analysing the entire pathway in Fig. 1, not just the part discussed above) show that almost all the metabolites shown are affected by stoichiometric constraints, the only exceptions being glucose, glycerol and pyruvate. However, glucose is limited by a different kind of constraint, such that its concentration cannot rise above the concentration in the host bloodstream, and glycerol appears to equilibrate with the bloodstream glycerol. This leaves just pyruvate, and leads to the conclusion that instead of 20 enzymes or transporters that can be regarded as suitable targets for drugs that act in the same sort of way as glyphosate, there is in reality just one, pyruvate transport (Eisenthal & Cornish-Bowden, 1998; Cornish-Bowden & Eisenthal, 2000). We believe this to be an important conclusion with practical implications for drug development that could not easily be reached by simple inspection of the metabolic pathway without stoichiometric analysis. Some highly relevant experimental data were published several years ago but unfortunately overlooked in our earlier work: Wiemer *et al.* (1995) found that α -cyano- β -(1-phenylindol-3-yl)-acrylate ("uk5099"), a powerful inhibitor of pyruvate transport, caused pyruvate to accumulate in cells of the bloodstream form of *T. brucei*, resulting in acidification of the cytosol, osmotic destabilization of the cells, and ultimately death. Thus, quite different arguments from ours led to the same conclusion, that "the pyruvate carrier is of vital importance for the bloodstream-form trypanosome".

In summary, we consider that algebraic analysis is necessary for revealing properties of pathways that could not be deduced just by inspection, and that the properties thus revealed supply knowledge necessary for at least three kinds of applications: computer simulation of pathways, deducing the existence of anaplerotic modifications of cycles to circumvent the constraints that would otherwise exist, and a more rational approach to drug design.

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