Metabolic Analysis in Drug Design: Complex, or Just Complicated?

Athel Cornish-Bowden and María Luz Cárdenas

CNRS-BIP, 31 chemin Joseph-Aiguier, B.P. 71,13402 Marseille Cedex 20, France
E-Mail: athel@ibsm.cnrs-mrs.fr

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Abstract

The metabolism of living organisms is certainly complicated, but it does not follow from this that it is complex, which would mean that its behaviour could not be computed, even in principle. For a simple organism like the parasite *Trypanosoma brucei,* the kinetics of glycolysis can in fact be computed with good accuracy from the known kinetic properties of the component enzymes. There may thus be no complexity to confront in the design of molecules intended to act as drugs. On the other hand, successful drug design will require much more attention to the functions of the intended targets than is evident in current practice, which is overwhelmingly structure-based. This will involve recognizing the different kinds of inhibition possible in a complete system and analysing the stoichiometric constraints that limit the variations in metabolite concentrations that are possible.

Introduction

Many properties of living systems are complicated, in the sense that the total behaviour can only be accounted for by taking account of many components. Complexity, however, is more than that, implying that the total is in some sense more than the sum of the parts. Rosen (1), for example, defines complexity in the following words: “I have attempted to introduce, and to motivate, a concept of complexity. A system is called complex if it has a nonsimulable model. Above all, complex systems cannot be completely chararacterized in terms of any reductionistic scheme based on simple systems.” Other definitions of complexity exist, for example that of von Neumann, but these are quite similar in meaning and there is a large degree of agreement in modern complexity theory that complexity is something along the lines indicated by Rosen. He goes on to argue that living systems are complex in just this sense, i.e. that a complete

description of a living system is, and will always be, beyond the reach of computation. This idea can be traced back to Schrödinger (2), who believed that the study of life would lead to new physical laws, or in other words that the laws of physics as determined from the study of non-living systems would be inadequate to describe life.

Monod (3) and other authors have seen suggestions of vitalism in this idea, so it is important to emphasize that Schrödinger's view is not in any sense vitalism. The essential distinction was made by Rashevsky (4), who pointed that the fact that life is constrained by the laws of physics does not require the existence of life to be predictable from the laws of physics. This parallels the idea that all of chemical kinetics obeys the laws of thermodynamics, but the laws of thermodynamics do not predict kinetic properties; yet a proposition that is regarded as uncontroversial and even trite when comparing thermodynamics with kinetics stimulates accusations of vitalism when it is applied to physics and biology.

Nonetheless, the biological revolution of the past half-century is the fruit of a resolutely reductionist approach, rejecting any idea of "new physics" and assuming that all the properties of living systems can be explained in terms of the physical properties of their components. Moreover, as Savageau (5) pointed out, "any respectable reductionist is also a reconstructionist", by which he meant that once one has characterized all the components of a system one ought to show that the whole system can be reconstructed by putting them all together, adding that "the problem is that the reconstructionist phase is seldom carried out." In this article, therefore, we shall examine a metabolic system, that of glycolysis in *Trypanosoma brucei,* where not only have the individual enzymes been well characterized kinetically, but the properties of the entire system can indeed be calculated from the properties of the components. If this is typical then it suggests that even though drug design may be complicated, requiring many different things to be taken into account, it is not complex. However, even if this is agreed, it does not justify using the same word to mean something else. Using "complex" to mean complicated is ultimately as confusing as it would be if biochemists agreed that vitalism in the 19th century sense had no part to play in biochemistry, but then decided to use the word "vitalism" to mean enzyme catalysis.

A separate problem derives from the fact that although lip-service is paid to structure-function relationships in attempts to rationalize drug discovery, the effort in practice is overwhelmingly devoted to structure, i.e. to the search for molecules with structures complementary to the known structures of biological molecules. Function is, if not completely forgotten, then at least

given no emphasis, and metabolism, an essential aspect of biological function, may pass unmentioned in entire issues of journals devoted to drug discovery (6), or mentioned only rarely, and then only in the context of the metabolic transformation of drugs, ignoring the metabolic functions of the drug targets (7). Although biotechnology is often presented as if progress in the past two decades represented a major success, the reality is different. For example, ten major classes of antibiotics were discovered between 1935 and 1963, but after 1963 there has been just one, the oxazolidones. This sort of observation explains the pessimism of some recent commentators (8, 9) on the state of the drug industry.

To illustrate the possibilities of doing better by taking account of the real behaviour of metabolic systems, we can examine how one might modify the activity of an enzyme in the cell (for example by genetic manipulation, or by the action of an inhibitor, etc.) to satisfy a technological aim. For example, if the objective is to eliminate a pest, one might suppose that the effect of an inhibitor could be to depress an essential flux to a level insufficient for life, or to raise the concentration of an intermediate to a toxic level. The former may seem the more obvious, but the latter is easier to achieve in practice, and there are some excellent examples of industrial products that work in that way, such as the herbicide glyphosate and antimalarials of the quinine class. A study of glycolysis in the parasite *Trypanosoma brucei* (which causes African sleeping sickness) indicates that for this approach to work the selected target enzyme must have a substrate with a concentration that is not limited by stoicheiometric constraints. That is not necessarily easy to find in a complicated system, and typically needs the metabolic network to be analysed in the computer.

IMPORTANCE OF INHIBITION TYPE

A high proportion of drug targets are enzymes, and in consequence a high proportion of drugs are enzyme inhibitors. The question therefore arises of how different types of enzyme inhibition affect the potential for pharmacological effects. From the kinetic point of view linear inhibitors range from competitive to uncompetitive inhibitors, separated by a large class of mixed inhibitors (including pure non-competitive inhibitors, the special case with identical competitive and uncompetitive components). This classification is complicated by the fact that irreversible inhibitors are sometimes confused with pure non-competitive inhibitors, because when added in doses insufficient to inactivate the enzyme completely they decrease the concentration of active enzyme while leaving the properties of the still-active molecules

unchanged. Tight-binding inhibitors are also easy to confuse with pure non-competitive inhibitors because whatever the true inhibition type the inevitable slowness of the inhibitor-release step means that in experiments on a short time scale they are indistinguishable from irreversible inhibitors. This classification is illustrated in Fig. 1.

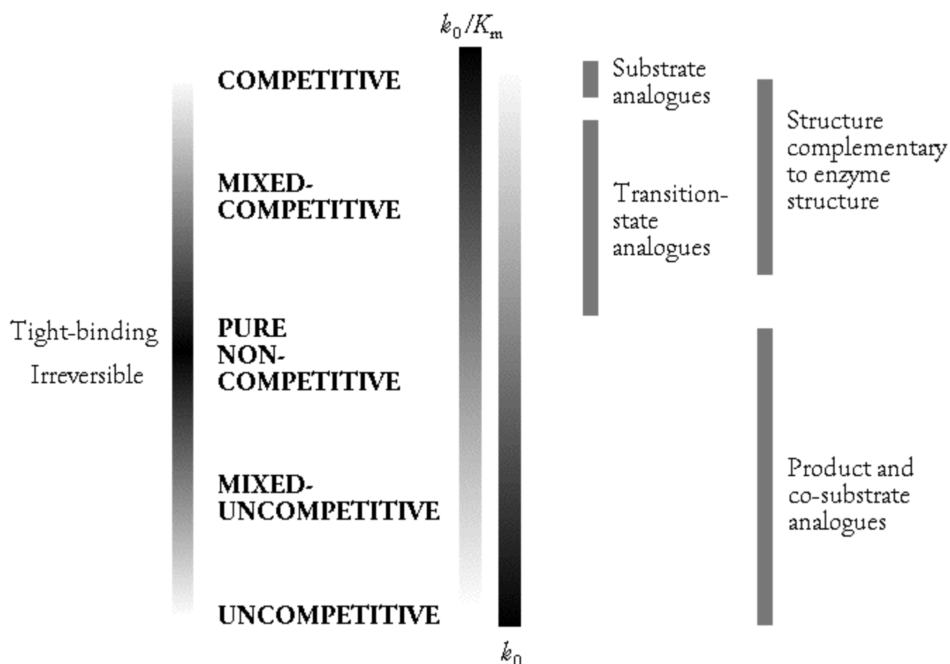

Figure 1. Classification of inhibitors. From the kinetic point of view simple inhibitors range from competitive, with effects only on the apparent value of the specificity constant k_0/K_m, to uncompetitive, with effects only on the catalytic constant k_0. There is a large class of mixed inhibitors with effects on both, including pure non-competitive inhibition, in which the competitive and uncompetitive components are equal. Tight-binding inhibitors may in principle fall into any of these types, but in practice are often made difficult to distinguish from pure non-competitive inhibitors by the very slow release of inhibitor in what is theoretically a reversible binding. Similar considerations apply to true irreversible inhibitors. The Figure also illustrates the types of inhibitory behaviour expected for various kinds of inhibitor that can be designed on the basis of structural considerations.

The inhibition types of various molecules likely to arise from purely structural considerations are also shown in the figure, but before discussing these we need to examine how different kinds of inhibition affect the kinetic behaviour of a living cell.

The first and possibly most important point is that there may be no easily observable effect at all unless the enzyme activity is decreased by a large factor, and perhaps not even then if the metabolic function of the inhibited enzyme can be replaced by an alternative enzyme or series of reactions. The typical form of the dependence of metabolic flux on the activity of any enzyme is as illustrated in Fig. 2, and in practice most enzymes are located in normal conditions near or to the right of the point labelled B, not near the point labelled A; in other words variation of an enzyme activity around its normal value will typically have little or no effect on the metabolic

flux. This is not merely a theoretical expectation (10) but it is also confirmed by numerous experimental studies (11, 12).

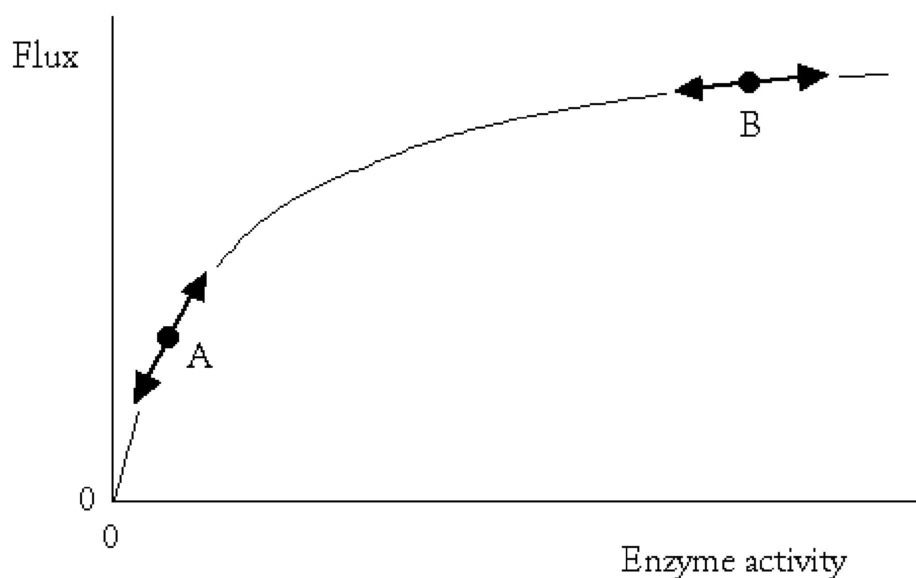

Figure 2. Dependence of metabolic flux on enzyme activity. The curve shown is typical of those found in numerous experimental cases (11, 12), and in the normal state nearly all enzymes are located near or to the right of the point labelled B; extremely few are found near the point labelled A. This implies that varying an enzyme activity *in vivo* will typically have no perceptible effect on the metabolic flux.

Many gene knock-out studies have produced no observed phenotypic effects: in *Saccharomyces cerevisiae,* for example, more than 80% of genes are “silent” (13), in the sense that any of them can be suppressed with no effect on growth or other gross aspects of the phenotype. Although this sort of result apparently surprised many observers it was entirely predictable and expected, as it follows almost automatically from results from metabolic control analysis that have now been in the literature (10) for nearly 30 years. The essential point is that a typical enzyme has a flux control coefficient close to zero for a gross phenotypic property like growth, and even for a more specific property such as the flux through the pathway in which the enzyme is located the flux control coefficients of most enzymes are small. This means that even if an enzyme is known to play an essential role in processes relevant to a particular disease, there is no certainty that inhibiting it will have a significant effect on the disease unless the inhibition is very strong (For activators the situation is even worse: as flux control coefficients normally decrease when the activity of an enzyme is increased, it is rare almost to the point of non-existence for activation of an enzyme *in vivo* to have a perceptible effect on the flux through the enzyme.).

The common inhibition types are easily confused in experiments in the spectrophotometer, with the result that cases of mixed inhibition are frequently reported as competitive. However,

ordinary steady-state experiments, typically done at substrate and product concentrations decided and fixed by the experimenter, are very misleading as a model of inhibition *in vivo,* where concentrations are not fixed at all, and certainly not by an external agent such as an experimenter. For a typical enzyme that catalyses a reaction in the middle of a metabolic pathway it is a better approximation (though still not exact) to consider that the rate is fixed and that the substrate and products are adjusted by the enzymes that use them to whatever values will sustain the appropriate flux. In these conditions competitive and uncompetitive inhibition become very different from one another (14), and the uncompetitive component becomes the main determinant of the response of the system to a mixed inhibitor.

These points are illustrated in Fig. 3 for the system of ten enzymes shown in Fig. 3a. When the inhibition is competitive (Fig. 3b) effects on both flux and metabolite concentrations are very slight, but all become much larger when the inhibition is uncompetitive (Fig. 3c).The essential point is that a molecule that competes with a substrate is a molecule that a substrate can compete with, and so the effect of a competitive inhibitor can be nullified by relatively minor adjustments of the concentrations around the inhibited enzyme. By contrast, effects of uncompetitive inhibitor are potentiated by the variations in substrate that they generate, and fairly modest levels of inhibition may therefore produce huge changes in substrate concentration. It is essentially this kind of effect that is exploited by glyphosate, an inhibitor of 3-phosphoshikimate 1-carboxyvinyltransferase, uncompetitive with respect to 3-phosphoshikimate (15).

Although the results of Fig. 3 are quite general for the effects of inhibiting an enzyme in the middle of a pathway, not all enzymes are located in the middles of pathways, and circumstances exist in which the illustration may be misleading. If an enzyme catalyses the first step in the transformation of a substrate such as glucose that is maintained at a stable and relatively high concentration by regulatory mechanisms independent of the pathway of interest, then it will resemble an enzyme in a spectrophotometer with a fixed substrate concentration rather than the sort of enzyme considered in Fig. 3. Another complication can arise when certain metabolite concentrations are constrained to remain within definite limits by stoichiometric relationships. Nonetheless, there remain many enzymes for which Fig. 3 gives a realistic picture of the likely effects of inhibition.

We now return to the part of Fig. 1 that is concerned with enzyme inhibitors designed on the basis of structural considerations, i.e. substrate analogues, transition-state analogues, molecules

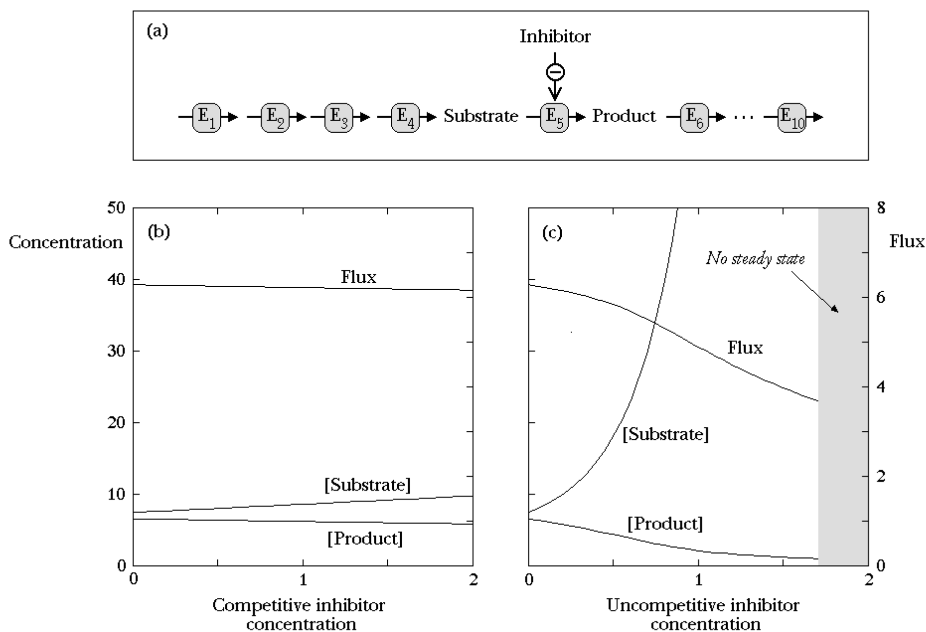

Figure 3. Effects of inhibition on an enzyme in the middle of a long pathway. (**A**) The pathway includes an enzyme E_5 that is the fifth of a series of ten enzymes and is acted on by an external inhibitor. Details of the assumptions used are given elsewhere (21); qualitatively they do not affect the results. (**B**) If the inhibition is competitive the effects both on the flux through the pathway and on the concentrations of the substrate and product of the inhibited enzyme are slight if the concentration of inhibitor does not become very large compared with the inhibition constant. (**C**) However, if the inhibition is uncompetitive the effects, especially on the substrate concentration become much larger, and can result in complete loss of the steady state. In (**B**) and (**C**) the inhibitor concentrations are given relative to the inhibition constants.

with structures complementary to parts of the enzyme, and analogues of products or co-substrates. Substrate analogues provide an easy choice: it is sufficient to find a molecule that resembles the substrate sufficiently to have similar binding properties, but which lacks a property essential for reaction to occur, as in the classic example of malonate, a structural analogue of succinate that acts as a competitive inhibitor of succinate dehydrogenase. Unfortunately, however, such a molecule is almost certain to act as a simple competitive inhibitor and is unlikely to bind appreciably more tightly than the substrate. As Fig. 3b shows, therefore, the easiest solution is unlikely to be the best.

A transition-state analogue is normally also predominantly competitive, though it may have significant uncompetitive character if the substrate of interest is the second or later substrate to bind in a sequential process. Moreover, it also often binds much more tightly than the substrates, and so it may be possible to deliver it to the binding site at a concentration much higher than its inhibition constant, and a significant effect can then be expected even for a competitive inhibitor. Molecules designed to bind to specific structural features of a target enzyme will in general have similar characteristics to transition-state analogues, with the same advantages and

disadvantages. However, careful choice of a molecule that can bind when the substrate is bound but not otherwise may in principle result in an uncompetitive inhibitor, which may be expected to have a large pharmacological effect, but we are not aware of any examples where this has been done in practice: the need for the inhibitor to bind only when the substrate is bound is commonly ignored in attempts to design drugs.

The final case to be considered is the one exemplified by the herbicide glyphosate (often sold as "Roundup"), whose chemical name is *N*-phosphonomethylglycine. Comparison of its structure with those of 3-phosphoshikimate and phospho*enol*pyruvate (Fig. 4), the two substrates of 3-phosphoshikimate 1-carboxyvinyltransferase, makes it clear that it is not a structural analogue of the first of these, though it could more plausibly be regarded as an analogue of the second. Kinetically, it does not bind to the free enzyme but it does compete with phospho*enol*pyruvate for the enzyme-3-phosphoshikimate complex. The authors who reported this finding found it surprising, and noted that corresponding competition is *not* found between *N*-phosphonome-thylglycine and phospho*enol*pyruvate in other apparently analogous cases, such as pyruvate kinase (15).

The example thus illustrates several points relevant to the search for uncompetitive inhibitors. A molecule that does not resemble the metabolite considered to be the substrate for the target enzyme but which does resemble one of its co-substrates may well prove to be uncompetitive with respect to the substrate of interest. (Of course, in the context of enzyme mechanisms there is no difference between a substrate and a co-substrate. Each is as much a substrate as the other. However, in the metabolic context the distinction is both commonplace and, usually, meaningful.).

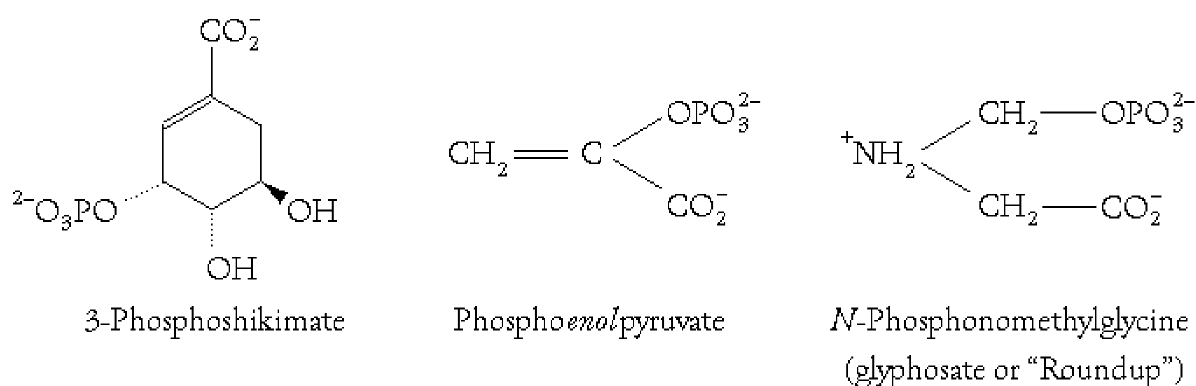

Figure 4. Comparison of the structure of *N*-phosphonomethylglycine (glyphosate, or "Roundup") with those of the two substrates of 3-phosphoshikimate 1-carboxyvinyltransferase.

Unfortunately, however, the co-substrate of any particular target enzyme will often be a metabolite like NAD, ATP or, as in this case, phospho*enol*pyruvate, that is also a substrate for other enzymes. Designing an uncompetitive inhibitor as a close structural analogue of a co-substrate then incurs the risk that it will lack specificity: even if it has the desired effect on the target enzyme it will also have similar but undesired effects on other enzymes. *N*-Phosphonomethylglycine then appears as a very fortunate case: similar enough to phospho*enol*pyruvate to interact strongly with one enzyme, but not similar enough to interact with others.

Designing an inhibitor with significant and specific uncompetitive character is thus a much more difficult task than designing a competitive inhibitor, because it cannot just be a substrate analogue. This difficulty is not an adequate reason for not attempting it, however, because solving a difficult task is likely to be more rewarding than solving an easy task if its solution is potentially useful and the solution to the easy problem potentially useless.

SYSTEMIC CONSIDERATIONS

It should never be forgotten that apart from a few secreted enzymes like invertase, enzymes do not act *in vivo* in isolation but as components of systems. This is the crucial point that explains why experiments in the spectrophotometer are often a poor guide to what is likely to happen *in vivo.* It is essential for considering fluxes, because the low flux control coefficients of most enzymes mean that it is normally very difficult to decrease a flux significantly by inhibition, and almost impossible to increase one by activation or overexpression (Fig. 2). Even when the objective is not to vary a flux but to vary a metabolite concentration the systemic context of the inhibition remains relevant because there are at least two circumstances where uncompetitive inhibition may not be much more effective than competitive. One of them we have already mentioned: an enzyme that acts on glucose at the beginning of a minor pathway, for example, will have very little effect on the glucose concentration in any ordinary conditions, because that is determined by controls on the major glucose-using pathways like glycolysis and glycogen synthesis; such an enzyme can therefore be treated like an enzyme in a spectrophotometer, and will respond to competitive inhibition as readily as to uncompetitive inhibition.

The other point is that a metabolite concentration can only show a large response to changes in the activities of enzymes that consume it or produce it if it is largely free from stoichiometric constraints. Some constraints are obvious from inspection: for example, in a cell with a fixed

total NAD concentration the concentrations of neither reduced nor oxidized NAD can exceed the fixed total. However, much more complicated constraints may also exist, and identifying these may require stoichiometric analysis by computer.

SIMULATING THE METABOLISM OF *TRYPANOSOMA BRUCEI*

The bloodstream form of the parasite *Trypanosoma brucei* has an unusually simple metabolism, and most of its metabolic activity is shown in Fig. 5. As most of the enzymes and transporters involved have been purified and their kinetic behaviour thoroughly characterized, it was possible to set up a detail kinetic model of the system as illustrated, and this was done by Bakker and colleagues (16). Both they and later we (17) found that the computer model was able to reproduce the known behaviour of the living organism with a fair degree of accuracy, and our suggestion on the basis of the model that inhibiting export of pyruvate would be lethal to the parasite proved (unknown to us at the time) to correspond to reality (18). All of this suggests that trypanosomal metabolism may be complicated (at least compared with ordinary kinetic experiments in the spectrophotometer even if it is far from complicated by comparison with other organisms), but that it is not complex.

As the pyruvate transporter is probably not the most obvious choice of target for drug design, given the nearly 20 other apparently reasonable targets in the metabolic scheme, we shall briefly indicate how it followed from the computer analysis. It is obvious from inspection of Fig. 5 that there are three simple conservation constraints, representing the sum of the two forms of NAD in the glycosome, the sum of the three adenine nucleotides in the glycosome, and the sum of the three adenine nucleotides in the cytosol. These last two are separate because these species do not cross the glycosomal membrane. In addition to these three constraints there is a fourth, which was identified by computer analysis; this involves all the phospho-groups in the glycosome that are not accounted for by entry of inorganic phosphate or exit of 3-phosphoglycerate, as well as two additional phosphorylated intermediates that are partitioned between the glycosome and the cytosol (19). In Fig. 5 these phospho-groups are marked in such a way as to make the conservation as obvious as possible, but if the structures are shown in a more conventional way the conservation relationship is far from obvious.

It follows from this analysis that in *Trypanosoma brucei* nearly all of the metabolites in the whole system participate in conservation relationships.

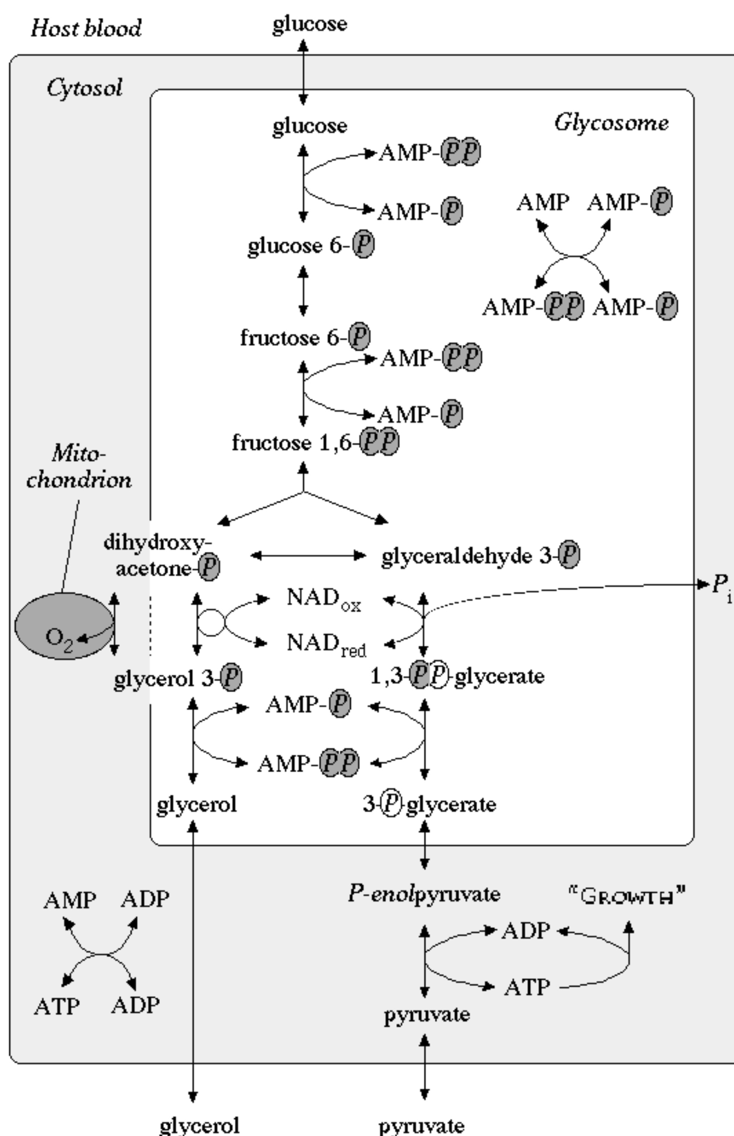

Figure 5. The glycolytic pathway in bloodstream form *Trypanosoma brucei.* There are four compartments, labelled Host blood, Cytosol, Glycosome and Mitochondrion. Dihydroxyacetone phosphate and glycerol 3-phosphate diffuse between the glycosome and the cytosol, but the two transport steps are not explicitly shown; glycerol 3-phosphate is reoxidized under aerobic conditions to dihydroxyacetone phosphate on the membrane of the mitochondrion. "GROWTH" represents all of the steps in the rest of metabolism that are driven by dephosphorylation of ATP. The phospho groups involved in the complicated conservation relationship are shown as *P* against a shaded background; the two unconserved phospho groups in the glycosome are shown against a white background.

This means that their concentrations cannot be changed by large amounts by altering enzyme activities, and this in turn means that extremely few of the enzymes and transporters in the system are plausible targets for a drug intended to act like glyphosate in plants. One of these few is the pyruvate transporter, and it appeared to be the only one that could not be eliminated for other reasons (17). Thus despite the apparent multitude of plausible drug targets in Fig. 5, there is only one that survives a closer analysis of the system.

DISCUSSION

The model of *Trypanosoma brucei* that we have considered is far from being a complete model of a living organism, as it ignores many processes, such as protein synthesis, cell division etc. that are certainly essential for the life of the organism even if they account for a relatively minor part of the metabolic activity of the parasite. Unfortunately this is no less true of all other metabolic models currently being studied, most of which include less experimental information and cover a much smaller proportion of the total metabolism of the cell than the *Trypanosoma brucei* model does. The question therefore remains open as to whether setting up a model that will allow computer simulation of an entire organism is possible, even theoretically, and thus whether living systems are truly complex in the sense used in complexity theory. However, both our results (17) and those of others (16) indicate that a large part of the trypanosomal metabolism that is likely to be of interest for drug design can certainly be simulated, with computer-generated results that correspond closely with experimental observations. In this limited sense, therefore, trypanosomal metabolism is not complex, and we expect that this will prove to be true for many other cases of pharmacological interest, and thus there will be no complexity to confront. On the other hand there are certainly complications that need to be taken into account, and progress in drug design will continue to be slow until the existence of enzymes *in vivo* as components of systems and not as entities on their own is generally recognized. This was understood by Wright (20) as long ago as 1934, but remains poorly understood by the world at large even today.

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