

## Prospects for Antiparasitic Drugs

THE CASE OF *TRYPANOSOMA BRUCEI*, THE CAUSATIVE AGENT OF AFRICAN SLEEPING SICKNESS\*

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**Glycolysis in the bloodstream form of *Trypanosoma brucei* provides a convenient context for studying the prospects for using enzyme inhibitors as antiparasitic drugs. As the recently developed model of this system (Bakker, B. M., Michels, P. A. M., Opperdoes, F. R., and Westerhoff, H. V. (1997) *J. Biol. Chem.* 272, 3207–3215) contains 20 enzyme-catalyzed reactions or transport steps, there are apparently numerous potential targets for drugs. However, as most flux control resides in the glucose-transport step, this is the only step for which inhibition can be expected to produce large effects on flux, and in the computer model such effects prove to be surprisingly small (although larger than those obtained by inhibiting any other step). It follows that there is little prospect of killing trypanosomes by depressing their glycolysis to a level incapable of sustaining life. The alternative is to use inhibition to increase the concentration of a metabolite sufficiently to interfere with the viability of the organism. For this purpose, only uncompetitive inhibition of pyruvate export proves effective in the model; in all other cases studied, the effects on metabolite concentrations are little more than trivial. This observation can be explained by the fact that nearly all of the metabolite concentrations in the system are held within relatively narrow ranges by stoichiometric constraints.**

Detailed information exists about metabolism in a wide variety of organisms, but there has been little success in designing molecules *ab initio* to realize specific metabolic objectives. For drugs intended to correct metabolic deficiencies, this is perhaps not surprising, but even drugs intended to eliminate particular pathogenic organisms (in principle, a much easier objective, as there must be many different ways of killing a healthy organism, even if there are extremely few ways of restoring an unhealthy one to health) mostly have rather broad specificity or they have been found more by chance than design.

In this paper, we examine why the level of success has been so low, taking the African trypanosome *Trypanosoma brucei* as an example. It is attractive for study because it is the causative agent of a major disease, African sleeping sickness, because its metabolism has been well studied, and because the kinetic properties of its glycolytic enzymes are known in as much

detail as is available for any organism, thanks mainly to the efforts of Opperdoes and colleagues (for reviews, see Refs. 1–4). The glycolytic pathway is an attractive target because the predominant bloodstream form of *T. brucei* has no energy resources, and relies entirely on rapid glycolysis for its energy supply. Indeed, the ability to kill trypanosomes by halting glycolysis was demonstrated 20 years ago (5, 6). Glycolysis in *T. brucei* has recently been the subject of a detailed kinetic model (7) that allows numerical testing of the likely metabolic consequences of inhibiting particular enzymes. Such testing is apparently not usually done, partly because suitable models are not available for many other organisms, but more particularly because it is commonly regarded as unnecessary, as it is assumed that delivering a specific inhibitor of a selected enzyme to the appropriate location will produce metabolic effects.

This attitude is well illustrated by a recent supplement to *Nature* entitled "Intelligent Drug Design"; this contains a general introduction (8), as well as articles on combinatorial chemistry (9, 10), improvements in screening procedures (11), antisense oligodeoxynucleotides (12), and methods of determining three-dimensional structures (13). These are important aspects of drug design, but the entire supplement neither mentions the words "metabolic" and "metabolism" nor shows any recognition that intelligent drug design must involve some consideration of the metabolic effects of the drugs once they have been designed.

The easiest inhibitors to design are substrate analogs, because they are likely to bind specifically to the active sites of the same enzymes as the substrates they resemble, and commonly act as competitive inhibitors. Unfortunately, as a strategy for designing pharmacologically active molecules, this approach is doomed to almost certain failure, because most such molecules do not bind much tighter than the substrates, and so it is difficult or impossible to deliver them to the target enzyme at concentrations that greatly exceed their inhibition constants. Concentrations similar to or lower than the inhibition constants are almost totally ineffective, because an organism can easily overcome competitive inhibition by increasing the substrate concentration enough to restore the rate to that in the absence of the inhibitor (14); the increase in substrate concentration necessary for this is typically around 2-fold.

There are two basic metabolic methods of killing an organism. Either the flux through an essential metabolic pathway can be decreased to the point where life is no longer possible, or a metabolite concentration can be increased to toxic levels. Although both of these are likely to involve enzyme inhibition, they are not the same, because depressing fluxes need not be accompanied by large changes in metabolite concentration, and enormous increases in metabolite concentration can occur with almost imperceptible changes in flux. The former will normally require a tight-binding inhibitor of an enzyme with a significant flux control coefficient, whereas the latter is most effective

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tively achieved with an uncompetitive inhibitor of an enzyme with a small flux control coefficient. Many successful pesticides and drugs are tight-binding inhibitors, but these are difficult to design because of the need to deliver and maintain concentrations at least 1000-fold higher than the inhibition constants. A few pesticides are uncompetitive inhibitors, the best-known example being the herbicide *N*-phosphonomethylglycine, commonly known as glyphosate or Roundup, an uncompetitive inhibitor of 3-phosphoshikimate 1-carboxyvinyltransferase (15).

The model of trypanosomal glycolysis developed by Bakker *et al.* (7) provides an excellent starting point for assessing effects of inhibition in whole pathways, as not only is a large amount of detailed kinetic information embedded in it, but also because the model gives an excellent account of the known properties of intact trypanosomes. One apparent exception to this generalization, the prediction that there is significant glycerol production under aerobic conditions despite some experimental suggestions to the contrary, may, as discussed below, reflect problems with the experiments rather than with the model.

#### EXPERIMENTAL MODEL

**Stoichiometry**—Glycolysis occurs in trypanosomes, as illustrated in Fig. 1. Most of the reactions, together with those that transform dihydroxyacetone phosphate into glycerol, occur in a special organelle known as the glycosome. The stoichiometry implies the existence of four distinct pools of conserved metabolites, of which three are obvious from inspection; the sums [ATP] + [ADP] + [AMP] are constant both in the cytosol and in the glycosome, and the sum [NAD] + [NADH] in the glycosome is likewise constant. The fourth relationship, however, is not easy to deduce without mathematical analysis of the kind pioneered by Reder (16).

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

Here and elsewhere in this paper, the subscript *c* refers to concentrations of species in the cytosol, and concentrations without subscripts refer to species in the glycosome. The volume ratio  $\rho$  is the ratio of the cytosolic and glycosomal volumes, estimated to be 22.3 (7). It enters the relationship because, although in single-compartment systems one may loosely regard the total concentration as being conserved, it is really mass that is conserved. The simpler expression in single-compartment systems is allowed only because then there is an exact proportionality between concentration and mass.

As the conservation relationships are automatically recognized and taken into account by the modeling program (see below), one might regard them as a complication that did not require discussion. However, the fact that all of the metabolites in the glycosome, apart from glucose, glycerol, inorganic phosphate, and 3-phosphoglycerate, are subject to conservation constraints places severe limits on the enzymes that may be profitable targets for drug design, as we discuss below. It is important therefore to recognize the nature of the relationship shown in Equation 1. Although most of the phosphorylated metabolites in the glycosome form part of it, 3-phosphoglycerate does not, and 1,3-bisphosphoglycerate has a coefficient of 1 in the equation even though it contains two phosphate groups. In fact, the conserved moiety is that part of the internal phosphate pool that is not accounted for by entry of inorganic phosphate and export of 3-phosphoglycerate.

**Kinetics**—All of the processes in the model apart from the aldolase reaction and the consumption of ATP for growth (see below) are either equilibria or can be expressed by introducing the kinetic constants listed in Table I into the following generic equation (in a few cases with exponents or additional terms, as noted in the footnotes to the table).

$$v = \frac{V_+ S_1 S_2 - V_- P_1 P_2}{K_{s1} K_{s2} - K_{p1} K_{p2}} \left( 1 + \frac{S_1}{K_{s1}} + \frac{P_1}{K_{p1}} \right) \left( 1 + \frac{S_2}{K_{s2}} + \frac{P_2}{K_{p2}} \right) \quad (\text{Eq. 2})$$

Both in the table and throughout the paper, all rates (including

values of limiting rates  $V$ ) are expressed as dimensionless numbers by writing them relative to a standard value of 1 nmol min<sup>-1</sup>/mg of cell protein.

The rate of the aldolase reaction was given by Equation 3, with  $V_+ = 184.5$ ,  $V_- = 220$ ,  $K_{\text{Fru}P_2}$  as given by Equation 4,  $K_{\text{Gla}3P} = 0.067$  mM,  $K_{\text{DHAP}} = 0.015$  mM,  $K_{i,\text{Gla}3P} = 0.098$  mM.

$v =$

$$\frac{V_+ \frac{[\text{Fru}P_2]}{K_{\text{Fru}P_2}} - V_- \frac{[\text{Gla}3P]}{K_{\text{Gla}3P}} \frac{[\text{DHAP}]}{K_{\text{DHAP}}}}{1 + \frac{[\text{Fru}P_2]}{K_{\text{Fru}P_2}} + \frac{[\text{Gla}3P]}{K_{\text{Gla}3P}} + \frac{[\text{DHAP}]}{K_{\text{DHAP}}} + \frac{[\text{Fru}P_2]}{K_{\text{Fru}P_2}} \frac{[\text{Gla}3P]}{K_{i,\text{Gla}3P}} + \frac{[\text{Gla}3P]}{K_{\text{Gla}3P}} \frac{[\text{DHAP}]}{K_{\text{DHAP}}}} \quad (\text{Eq. 3})$$

$$K_{\text{Fru}P_2} = 0.009 \left( 1 \text{ mM} + \frac{[\text{ATP}]}{0.68} + \frac{[\text{ADP}]}{1.51} + \frac{[\text{AMP}]}{3.65} \right) \quad (\text{Eq. 4})$$

Utilization of ATP by the parasite for growth, etc., is assumed to depend on the ratio of ATP and ADP concentrations in the cytosol, with a rate of 50[ATP]/[ADP]<sub>c</sub>.

With three exceptions, these definitions result in kinetic equations identical to those given by Bakker *et al.* (7). For the mitochondrial oxidation of glycerol 3-phosphate, however, we have used the results of Eiseenthal and Panes (17) to include an explicit dependence of rate on oxygen concentration. Our equation gives exactly the behavior assumed by Bakker *et al.* (7) at the extremes of aerobiosis and anaerobiosis, but it also takes account of the observed effects of intermediate oxygen concentrations (17). In view of the considerations discussed below under "The Transition from Aerobic to Anaerobic Conditions," we also prefer not to assume zero activity for glycerol kinase in aerobic conditions, and thus use the kinetic constants given in Table I at all oxygen concentrations.

The third exception is pyruvate kinase, which Bakker *et al.* (7) treated as irreversible, with the kinetic constants shown in Table I, quite reasonably so in the conditions they studied, in view of the large equilibrium constant and the low steady-state concentration of pyruvate. However, as we shall be studying effects of inhibiting pyruvate export, and it is impossible for pyruvate to accumulate to very high levels without producing any mass action effects on the earlier steps, we need to allow for reversibility, however slight, of the pyruvate kinase reaction. We have therefore set the equilibrium constant  $K$  to  $3 \times 10^5$ , calculated from the value of  $\Delta G^{\circ} = -7.5$  kcal mol<sup>-1</sup> (18), and in the absence of experimental information have approximated  $K_{\text{pyruvate}}$  and  $K_{\text{ATP}}$  by  $K_{\text{PEP}}$  and  $K_{\text{ADP}}$ , respectively.

$$v = \frac{V \frac{[\text{PEP}]_c [\text{ADP}]_c}{K_{\text{PEP}} K_{\text{ADP}}} \left( 1 - \frac{[\text{pyruvate}]_c [\text{ATP}]_c}{K [\text{PEP}]_c [\text{ADP}]_c} \right) \left( \frac{[\text{PEP}]_c}{K_{\text{PEP}}} + \frac{[\text{pyruvate}]_c}{K_{\text{pyruvate}}} \right)^{h-1}}{\left\{ 1 + \left( \frac{[\text{PEP}]_c}{K_{\text{PEP}}} + \frac{[\text{pyruvate}]_c}{K_{\text{pyruvate}}} \right)^h \right\} \left( 1 + \frac{[\text{ADP}]_c}{K_{\text{ADP}}} + \frac{[\text{ATP}]_c}{K_{\text{ATP}}} \right)} \quad (\text{Eq. 5})$$

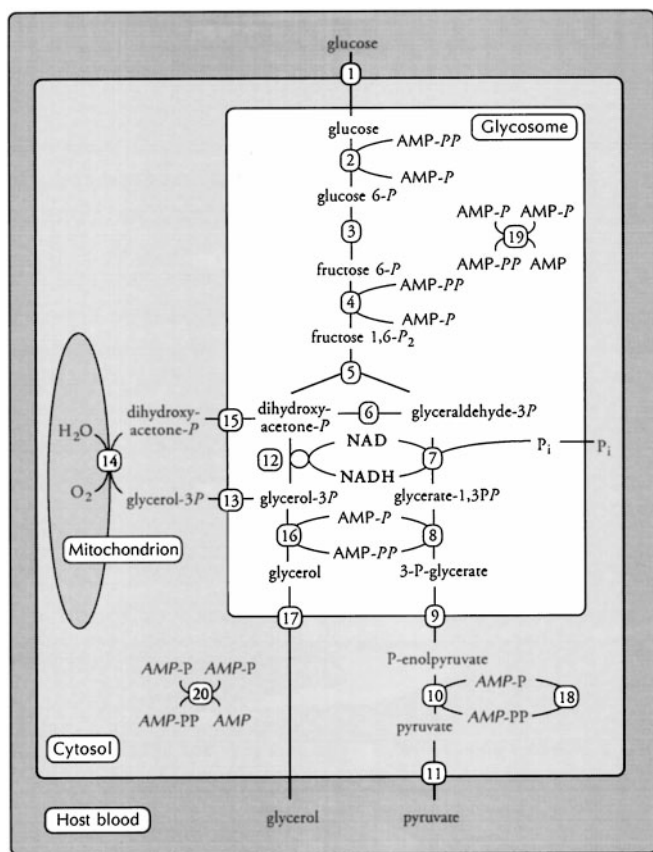
This equation gives exactly the kinetics defined in Table I when the two product concentrations are zero, and it gives a rate of zero under any equilibrium conditions. The need to satisfy both of these requirements simultaneously accounts for the apparently excessive complexity of the equation, which is written in accordance with the principles discussed elsewhere (19).

**Computation**—Steady states for the model as defined in the previous section were calculated by means of the program Gepasi 3.01, described (in an earlier version) by Mendes (20).<sup>1</sup> Bakker *et al.* (7) used two different programs: MLAB (Civilized Software, Bethesda, MD) and SCAMP (21, 22). We have checked that Gepasi gives the same numerical results as those reported (7) when given the same input. Initially, we attempted to use MetaModel (23), but this proved incapable of handling a model of the required complexity.

#### RESULTS

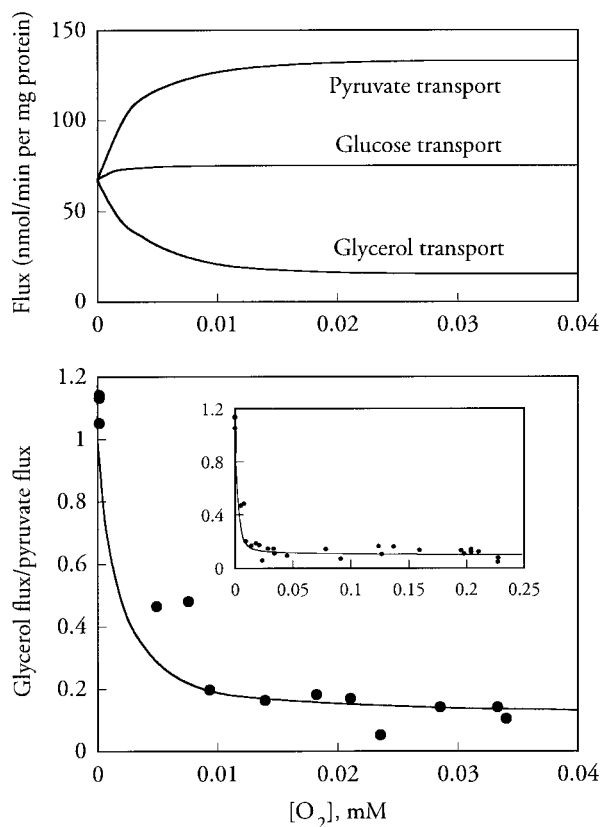
**Control Distribution**—Bakker *et al.* (24) reported that the greatest share of flux control in their trypanosome model (7) resides in glucose transport when the transporter has the level of activity measured in the bloodstream form of *T. brucei*, and

<sup>1</sup> The Gepasi software is available via the World Wide Web (<http://gepasi.dbs.aber.ac.uk/softw/gep3dwld.html>).



**FIG. 1. Compartmentation of trypanosomal glycolysis.** The enzymes and transporters are numbered as follows: 1, glucose transport; 2, hexokinase; 3, hexose-phosphate isomerase; 4, phosphofructokinase; 5, aldolase; 6, triose-phosphate isomerase; 7, glyceraldehyde-3-phosphate dehydrogenase; 8, 3-phosphoglycerate kinase; 9, phosphoglycerate mutase, enolase, and phosphoenolpyruvate transport (treated as a single process); 10, pyruvate kinase; 11, pyruvate transport; 12, glycerol-3-phosphate dehydrogenase; 13, glycerol-3-phosphate transport; 14, mitochondrial oxidation of glycerol 3-phosphate; 15, dihydroxyacetone phosphate transport; 16, glycerol kinase; 17, glycerol transport; 18, "growth" (all processes that convert ATP to ADP apart from those shown explicitly); 19, glycosomal myokinase; 20, cytosolic myokinase. This is essentially the model used by Bakker *et al.* (7), apart from the fact that we show the participation of inorganic phosphate in step 7, as well as its (presumed) equilibration across the glycosomal membrane. It is essential to make this participation explicit if one is to understand the nature of the most complicated of the four conservation relationships implied by the stoichiometry, which are as follows: (i) conservation of the AMP moiety (represented by *sans-serif type*) of ATP, ADP and AMP in the glycosome; (ii) conservation of the AMP moiety (represented by *italic sans-serif type*) in the cytosol; (iii) conservation of NAD + NADH (represented by *bold type*); and (iv) conservation of those phosphate groups (represented by *P in italics*) that are not accounted for by entry of inorganic phosphate and export of 3-phosphoglycerate. ATP and ADP are represented as AMP-PP and AMP-P, respectively, so as to allow the separate conservation of AMP and of phospho moieties in the glycosome to be recognized easily.

we have confirmed that this is correct. We have also examined the changes in control distribution that result from allowing for the reverse reaction catalyzed by pyruvate kinase. Although the fact that this reaction is far from equilibrium under all ordinary conditions would suggest that taking account of its reversibility would have a trivial effect, the change is rather more than trivial; the flux control coefficient for pyruvate transport is exactly zero in the conditions considered by Bakker *et al.* (7, 24), but it assumes the second largest value when the reverse pyruvate kinase reaction is taken into account. Nonetheless, it remains true that glucose transport has the largest share of flux control and that no other process has a major share.



**FIG. 2. Transition from anaerobic to aerobic conditions.** Three transport fluxes, and the ratio of the fluxes for glycerol and pyruvate, are shown as functions of the concentration of oxygen. All of the curves were calculated from the model as described in the text, but the experimental points are from Ref. 17; in other words, the curve in the lower part of the figure is not a best-fit curve but is calculated independently of the data. The inset shows the same comparison extended into the range of oxygen concentrations where the fluxes are virtually independent of oxygen concentration.

*The Transition from Aerobic to Anaerobic Conditions*—Bakker *et al.* (7) assumed that glycerol kinase had no activity in aerobic conditions. They introduced this discontinuity into their model to obtain a zero efflux of glycerol under those conditions. The question of glycerol production from glucose is one that has been addressed by several groups of workers. Simple consideration of the metabolic scheme shown in Fig. 1 might be taken as predicting the production of 2 mol of pyruvate/mol of glucose consumed under aerobic conditions, and 1 mol each of pyruvate and glycerol/mol of glucose used under anaerobiosis (1). However, glycerol/pyruvate ratios in the range 0.1–0.4 have been observed under aerobic conditions (17, 25–27). Similar results were found by Fairlamb and Bowman (28), who ascribed the presence of glycerol in aerobic incubations of trypanosomes with glucose to experimental artifacts. In all these investigations, measurements were performed under fully aerobic or fully anaerobic conditions, the latter including anaerobiosis simulated by inhibition of glycerophosphate oxidase by salicylhydroxamic acid. However, a systematic study (17) of the ratio of glycerol and pyruvate effluxes over a range of oxygen concentrations from zero to 0.25 mM showed that the efflux of glycerol does not fall to zero in aerobic conditions, but stabilizes at about 10% of the efflux of pyruvate, exactly as the model of Bakker *et al.* (7) would predict if glycerol kinase had the same activity in aerobic as in anaerobic conditions.

We have therefore made a detailed comparison between the experimental data and the model with glycerol kinase active, and find a high degree of agreement (Fig. 2). Even if the curve

TABLE I  
Kinetic constants

All constants refer to the generic equation shown in the text as Equation 2, the substrates and products being numbered in the order they appear in the equation that defines the stoichiometry of each process, e.g. for hexokinase,  $K_{s1}$  refers to ATP,  $K_{s2}$  to glucose, etc.

Process: $S_1 + S_2 = P_1 + P_2$	$V_+$	$V_-$	$K_{s1}$	$K_{s2}$	$K_{p1}$	$K_{p2}$
			<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
Glucose transport, <sup>a</sup> $Glc_x = Glc$	106.2	106.2	2		2	
Hexokinase, $ATP + Glc = ADP + Glc6P$	625	0	0.116	0.1	0.126	$\infty$
Hexose- <i>P</i> isomerase, $Glc6P = Fru6P$			Equilibrium with $P_1/S_1 = 0.29$			
Phosphofructokinase, <sup>b</sup> $Fru6P + ATP = FruP_2 + ADP$	780	0	0.82	0.026	$\infty$	$\infty$
Aldolase, $FruP_2 = Gla3P + DHAP$			See Equations 3 and 4 in the text			
Triose- <i>P</i> isomerase, $DHAP = Gla3P$			Equilibrium with $P_1/S_1 = 0.045$			
Glyceraldehyde 3- <i>P</i> dehydrogenase, $Gla3P + NAD = GbisP + NADH$	1470	980	0.15	0.45	0.1	0.02
3- <i>P</i> -glycerate kinase, $GbisP + ADP = 3PG + ATP$	640	18.5	0.05	0.1	1.62	0.29
<i>P</i> -Glycerate mutase, enolase, $PEP$ transport, $3PG = PEP_c$			Equilibrium with $P_1/S_1 = 1.25$			
Pyruvate kinase, <sup>c</sup> $PEP_c + ADP_c = Pyr_c + ATP_c$	2600	0	Note c	0.114	$\infty$	$\infty$
Pyruvate export, $Pyr_c = Pyr_x$	160	0	1.96		$\infty$	
Glycerol-3 <i>P</i> dehydrogenase, $DHAP + NADH = Glo3P + NAD$	425	29.8	0.85	0.015	6.4	0.6
Glycerol-3 <i>P</i> transport, $Glo3P = Glo3P_c$			Equilibrium with $P_1/S_1 = 1$			
Dihydroxyacetone- <i>P</i> transport, $DHAP = DHAP_c$			Equilibrium with $P_1/S_1 = 1$			
Respiration, $Glo3P + O_2 = DHAP$	368	0	1.7	0.008	$\infty$	
Glycerol kinase, <sup>d</sup> $Glo3P + ADP = Glo + ATP$	200	33400	5.1	0.21	0.12	0.19
Glycerol export, $glycerol = glycerol_x$			Equilibrium with $P_1/S_1 = 1$			
Growth			50[ATP] <sub>c</sub> /[ADP] <sub>c</sub> (see text)			
Myokinase, <sup>e</sup> $ADP + ADP = ATP + AMP$			Equilibrium with $P_1P_2/S_1S_2 = 0.442$			

<sup>a</sup> For glucose transport there is an additional term  $\alpha S_1 P_1 / (K_{s1} K_{p2})$  in the denominator of Equation 2, with  $\alpha = 0.75$ .

<sup>b</sup>  $S_1$  and  $K_{s1}$  are raised to the power 1.2 in Equation 2.

<sup>c</sup>  $S_1$  and  $K_{s1}$  are raised to the power 2.5 in Equation 2, and  $K_{s1}$  is given by  $0.34 \cdot (1 \text{ mM} + [ATP]_c/0.57 + [ADP]_c/0.64)$ . See also Equation 5 in the text.

<sup>d</sup> The same kinetics are assumed in both aerobic and anaerobic conditions (see text).

<sup>e</sup> The same equation applies to the independent myokinase equilibria in the glycosome and the cytosol.

were a best-fit curve, one could consider the fit quite adequate, but given that it is calculated independently from the data (apart from the fact that the same experimental data were the source of the  $K_{O_2}$  value defined in Table I), the fit is essentially perfect. With glycerol kinase active in all conditions, therefore, the model accounts not only for the behavior at the extremes of oxygen concentration but also for the transition between the two. It is a striking illustration of the robustness of the model that it proves able to predict behavior that was not taken into account during its construction.

Bakker *et al.* (7) partially justified their assumption that glycerol kinase is inactive under aerobic conditions by pointing out that a very low external glycerol concentration would be sufficient to inhibit glycerol efflux completely. We have confirmed that this is true, but point out that unless this external concentration happened to have precisely the right value it would bring the efflux not necessarily to zero but to some other value, which could be either positive or negative. At higher external glycerol concentrations, it can act as an alternative substrate to glucose for aerobic metabolism, not only in the model but also experimentally (27).

**Inhibition of Glucose Transport**—Bakker *et al.* (24) found the step with the highest degree of control over trypanosome growth to be glucose transport, and we have confirmed that this remains true if the reversibility of pyruvate kinase is taken into account, although its flux control coefficient in this case (0.34) is too small for it or any other reaction to be regarded as rate-limiting. It follows that, if the objective of designing a drug is to prevent trypanosomes from achieving a viable metabolic flux, then the only realistic candidate for the step to be inhibited is glucose transport, although it has so little control that one cannot be optimistic that a large depression of growth can be produced by inhibiting it. Inhibition of any other step can be expected to have even less effect on the metabolic flux unless the degree of inhibition is very high.

Use of the model to simulate the effects of inhibiting glucose transport confirmed these expectations; for example, the presence of a competitive inhibitor at a concentration equal to its inhibition constant, simulated by adding a term equal to unity

to the denominator of the rate equation, produced a flux through glucose transport of 79% of the uninhibited flux, but the growth flux, *i.e.* the net production of ATP, decreased only to about 85% of the uninhibited value.

This result has depressing implications for the prospects of lowering a flux to a non-viable level as a strategy for pest control. First, it is clear that for decreasing the flux only glucose transport will do; inhibiting any other step is likely to be futile unless the level of inhibition that can be achieved is high enough to eliminate the enzyme activity virtually completely and permanently. Second, the effects of inhibiting glucose transport are not impressive. Decreasing the growth flux by 15% is unlikely to eliminate the trypanosomes unless the inhibition can be maintained for a substantial period.

A mitigating feature may be that flux control coefficients do not remain constant when the conditions are altered, and, as Bakker *et al.* (24) pointed out, a step that appears to have insignificant control in normal conditions may acquire sufficient control in special circumstances to be a worthwhile target for inhibitors. However, it remains to be demonstrated whether this approach can work in practice, or even in the computer model.

**Inhibition of Pyruvate Transport**—An alternative strategy to decreasing the carbohydrate flux would be to cause a metabolite concentration to rise to catastrophic levels. At first sight, the fact that nearly all of the steps in the model have very little control over flux suggests that there are many candidates for enzymes that could be inhibited to produce an uncontrolled accumulation of their substrates. In fact, the choice is far more restricted than it may appear because the numerous conservation relationships mean that very few metabolite concentrations can rise in an uncontrolled manner.

Pyruvate transport is one of the few processes that is not involved in conservation relationships. Although it has a flux control coefficient of zero (24) in the model as originally described (7), this rises to about 0.2 when pyruvate kinase is made reversible. This is still low enough to suggest that it responds approximately to inhibition like a constant-flux enzyme. Earlier studies (14, 29) of such enzymes showed that

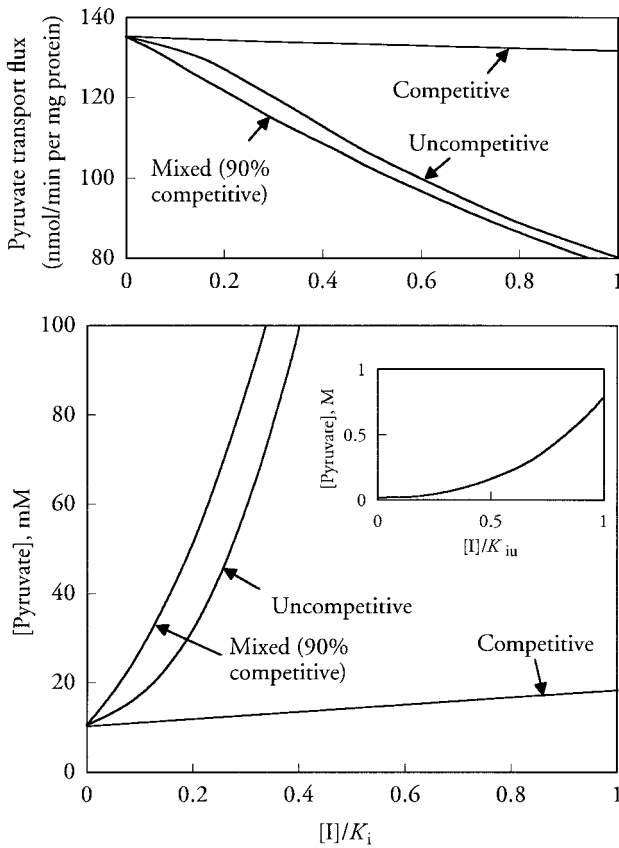


FIG. 3. **Effects of inhibiting pyruvate transport.** The pyruvate transport flux and the pyruvate concentration are shown as functions of inhibitor concentration, for three types of inhibition of pyruvate transport, as labeled, the case of mixed inhibition being one with  $K_{iu} = 10K_{ic}$ , i.e. the competitive component was 10-fold stronger than the uncompetitive component. For labeling the *abscissa axis*,  $K_i$  is taken to be the competitive inhibition constant  $K_{ic}$  for the case of competitive inhibition, but otherwise it is the uncompetitive inhibition constant  $K_{iu}$ .

competitive inhibition causes the substrate concentration to rise linearly, being doubled by an inhibitor concentration equal to the inhibition constant; by contrast, uncompetitive inhibition produces an infinite substrate concentration (i.e. no steady state exists) at a finite inhibitor concentration.

To put this more concretely, consider the inclusion in the kinetics of pyruvate transport of a term allowing for inhibition by a hypothetical uncompetitive inhibitor,  $I_u$ , with inhibition constant  $K_{iu}$ .

$$v = \frac{V[\text{pyruvate}]}{K_{\text{pyruvate}} + [\text{pyruvate}](1 + [I_u]/K_{iu})} \quad (\text{Eq. 6})$$

This may be transformed into the following form (cf. Equation 10.11 of Ref. 29) to give the pyruvate concentration as a function of inhibitor concentration when the flux is constant.

$$[\text{Pyruvate}] = \frac{K_{\text{pyruvate}}}{\frac{v}{V} - 1 - \frac{[I_u]}{K_{iu}}} \quad (\text{Eq. 7})$$

Because the computation indicated that this process is well over half-saturated, only a small value of  $[I_u]/K_{iu}$  is needed to drive the system into instability.

Now, it may be argued that impressive as this effect of uncompetitive inhibition may be it has little practical usefulness, as uncompetitive inhibitors are not common and are difficult to design. However, there is no objection to the presence of a competitive component, i.e. mixed inhibition will work just as well as uncompetitive, the only requirement being that

TABLE II  
Effects of inhibiting hexokinase

Inhibition conditions	[Glc]	[ATP]	[Glc6P]	[ADP]	Glucose transport	Growth flux
	mM	mM	mM	mM		
No inhibition	0.04517	1.011	0.4407	1.669	73.80	135.0
$t_{c,ATP} = 0.5$	0.04622	1.019	0.4404	1.670	73.75	134.9
$t_{c,ATP} = 10$	0.06533	1.179	0.4344	1.672	72.90	134.2
$t_{u,ATP} = 0.2$	0.04892	1.042	0.4395	1.672	73.63	134.8
$t_{u,ATP} = 20$	1.788	3.742	0.1838	0.1548	28.09	55.98
$t_{c,Glc} = 0.2$	0.05159	1.064	0.4386	1.673	73.51	134.7
$t_{c,Glc} = 10$	0.2180	2.323	0.3958	1.269	66.58	127.2
$t_{u,Glc} = 0.2$	0.04833	1.037	0.4397	1.671	73.66	134.9
$t_{u,Glc} = 20$	1.808	3.744	0.1820	0.1526	27.78	55.36

the inhibitor concentration must exceed some small factor of the inhibition constant for the uncompetitive component; whether or not a competitive component is also present, and its magnitude if it is present, is almost irrelevant.

As illustrated in Fig. 3, this simplified analysis based on the kinetics of one irreversible enzyme gives results that are approximately but not exactly correct. In the case of competitive inhibition of pyruvate transport, there is virtually no decrease in flux for  $[I]/K_{ic}$  values up to 1, and consequently the curve showing the dependence of the pyruvate concentration on the inhibitor concentration is indistinguishable by eye from a straight line. For other types of inhibition, the flux decreases appreciably as the degree of inhibition increases; consequently, although the pyruvate concentration does rise extremely steeply, the slope does not continue increasing indefinitely (see inset to Fig. 3). Indeed, the pyruvate concentration itself must eventually level out when the entire system approaches equilibrium. However, the equilibrium concentration of pyruvate is far higher than could ever be reached in practice, and so it remains true that even in a complete system with reversible kinetics inhibition by an uncompetitive or mixed inhibitor can produce toxic levels of substrate at low inhibitor concentrations. The model upon which this analysis is based is a metabolic one, and does not take into account transcription and translation. However, the high glycolytic flux in the predominant long slender (bloodstream) form of *T. brucei* means that metabolic effects will be manifested far more rapidly than events depending on protein synthesis. A similar conclusion is likely to apply also to possible host-parasite interactions, e.g. at the immunological level, but this will have to be confirmed by studies *in vivo*.

**Inhibition of Other Steps**—Inhibition of other steps produced much less dramatic results than those illustrated in Fig. 3, regardless of the type of inhibition. At first sight this was surprising, as the elementary theory (14) suggested that uncompetitive inhibition of any enzyme with little control over flux would produce very large increases in the substrate concentration. The results will be illustrated by some data for hexokinase, although similar ones were obtained with other apparently promising candidates, such as aldolase.

To allow for various possible modes of inhibition of hexokinase, its kinetic equation was rewritten as follows.

$$v = \frac{V \frac{[\text{ATP}][\text{Glc}]}{K_{ATP} K_{Glc}}}{\left[ 1 + t_{c,ATP} + \frac{[\text{ATP}](1 + t_{u,ATP})}{K_{ATP}} + \frac{[\text{ADP}]}{K_{ADP}} \right] \left[ 1 + t_{c,Glc} + \frac{[\text{Glc}](1 + t_{u,Glc})}{K_{Glc}} \right]} \quad (\text{Eq. 8})$$

In this equation  $t_{c,ATP}$ ,  $t_{u,ATP}$ ,  $t_{c,Glc}$ , and  $t_{u,Glc}$  are dimensionless inhibition terms allowing for competitive or uncompetitive inhibition with respect to ATP and with respect to glucose (each can be regarded as a concentration divided by the appropriate

inhibition constant). When all are set to zero, Equation 8 gives identical kinetics to Table I. Effects of assigning non-zero values are shown in Table II.

Although some non-trivial effects on both concentrations and the flux are visible in the table, they are far smaller than those seen in Fig. 3, and occur at much higher levels of inhibition, because none of the relevant concentrations can increase in an uncontrolled manner. The concentration of glucose 6-phosphate is constrained by Equation 1, and those of ATP and ADP are constrained both by Equation 1 and by the general conservation of adenine nucleotides. Their concentrations cannot therefore increase by more than small factors. Although glycosomal glucose does not appear in Equation 1 or the other conservation relationships, its concentration is also constrained by the fact that it cannot rise above the concentration of glucose in the host bloodstream, which means that it cannot increase by more than a factor of about 90. Analogous studies were also made of some other enzymes, such as aldolase, that appeared to have non-negligible flux control, with similar results.

#### DISCUSSION

Our analysis of effects of inhibiting particular steps in trypanosomal glycolysis has several implications for antimebolite drugs in general, because little of it depends on specific characteristics of the model considered. First of all, destroying a parasite by decreasing its metabolic flux to a level that cannot sustain life is a less attractive option than it may appear at first sight. Unless a specific irreversible inhibitor is available that can decrease an enzyme activity to zero, the effects of inhibition on flux are usually much smaller than studies on the isolated enzyme may suggest. This is because few enzymes have flux control coefficients close to unity, and the effect of a low inhibitor concentration on the flux in an integrated system is therefore always smaller than the effect on the isolated enzyme. Although the flux control coefficient of almost any enzyme increases as it is inhibited, the increase is gradual, and, as illustrated for glucose transport in the trypanosome, inhibiting even the enzyme with the highest flux control coefficient is likely to produce disappointing results.

Increasing the concentration of a metabolite to toxic levels by uncompetitive inhibition (or mixed inhibition with a perceptible uncompetitive component) is also not straightforward, for more than the obvious reason that uncompetitive inhibitors are not easy to find or design. Although this strategy works very effectively if applied to an appropriate enzyme or transporter (illustrated with pyruvate transport in Fig. 3), the choice of appropriate enzymes is far more limited than it may appear at first sight, because the inhibited step must have a substrate whose concentration is not limited by the stoichiometry of the network.

For the model illustrated in Fig. 1, such stoichiometric considerations exclude nearly all of the steps, because nearly all of the intermediates are involved in one or more conservation relationships. Even glucose, although not part of one of these relationships, is limited by the fact that its concentration in the glycosome cannot rise above the concentration in the host bloodstream. This last limitation will be even more severe in

other organisms, because few have glucose concentrations as low as that found in the glycosome of *T. brucei*, which has one of the highest levels of glycolytic activity known in eukaryotic cells.

It follows that analyzing the stoichiometric structure of a network is not just an abstract topic of mainly theoretical interest, but has major practical implications. Without carrying out the analysis, few would realize that the concentrations of nearly all of the metabolites in Fig. 1 are constrained by stoichiometric considerations. When the stoichiometric constraints are taken into account, the only realistic candidates for inhibition are pyruvate transport, glycerol transport, and the three reactions between 3-phosphoglycerate and phosphoenolpyruvate lumped together as step 9. The last two are ruled out by the fact that they are assumed to be at equilibrium, and glycerol transport is additionally ruled out by the fact that, under aerobic conditions, it has such a low rate of efflux that some authors have doubted whether it occurs at all. Fortunately, the one remaining candidate, pyruvate transport, proved to respond to inhibition as expected. Thus, there is good reason to believe that, if an inhibitor of pyruvate transport with a sufficient uncompetitive component could be found, it would be expected to have powerful anti-trypanosomal activity.

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