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PROSPECTS FOR PHARMACOLOGICAL MANIPULATION OF METABOLISM

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The development of our understanding of the kinetic behaviour of metabolic systems was dominated throughout most of its history by studies of single enzymes in isolation from others. Initially, of course, Buchner's "zymase" (Buchner, 1897) was believed to be a single enzyme, but once it was recognized to be a mixture there was no attempt to analyse the kinetic properties of the mixture as such; the drive was always to separate the components and study them separately.

The growth of interest in metabolic regulation after the discovery of feedback regulation during the 1950s (Umbarger, 1956; Yates and Pardee, 1956) changed this perspective surprisingly little. For a long time, the textbooks continued to discuss the regulation of pathways in terms of the one enzyme believed to be rate-limiting. This still remains largely true today, despite the recent development of more systemic approaches to metabolism, starting from the classic papers of Kacser and Burns (1973) and Heinrich and Rapoport (1974); thus Fell (1996) could remark in the Preface of his recent book that

> Criticisms and reworkings of traditional biochemical explanations are still not widely known and have, as yet, had little influence on the contents of the standard biochemical textbooks, which still cling to the rejected concepts, usually without even mentioning the doubts and problems.

There can be little doubt that at least part of the inertia is due to a perception that metabolic control analysis is excessively mathematical and that it is not closely enough linked with the real world in which

experimental studies of metabolic regulation are carried out. Actually, experimental work inspired by the ideas of control analysis has existed now for more than two decades, and the expression of the central ideas does not have to be heavily mathematical — Fell's book, for example, contains no more mathematics than one expects to find in any book on enzyme kinetics, and almost no matrix algebra at all.

Nonetheless, the doubt remains: is a mathematical treatment necessary for understanding metabolic regulation, and can computer studies of metabolic pathways tell us anything about them that is not obvious from inspection? Even if we can learn non-obvious facts from the computer, do these facts have any importance? Can knowledge of them help, for example, to develop medicine or biotechnology? In this chapter we intend to address these questions, and using the metabolism of *Trypanosoma brucei* as an example we shall try to show not only that computer analysis can reveal information that could not easily be recognized by inspecting a pathway, but also that such information may provide the *only* feasible route to the understanding that is needed for combatting a major disease.

Trypanosoma brucei is not only responsible for much human suffering as the causative agent of African sleeping sickness, but it also, because of its effects on cattle, has major economic consequences. Before considering it as a special case, however, it will be useful to look at drug development in a more general way, because there seems to be a widespread view that the metabolic effects of drugs are so obvious that they do not need to be taken into account in the early stages of drug design. For example, a special supplement of *Nature* (1996) devoted to "intelligent drug design" contains articles on combinatorial chemistry, improvements in screening procedures, antisense oligodeoxynucleotides and methods of determining three-dimensional structures, but nowhere mentions the words "metabolic" or "metabolism".

Yet what drugs do is alter metabolism, and to have any advance idea of the likely metabolic consequences of the presence of a particular agent in the body it is not sufficient to know that it activates or inhibits a particular enzyme. If, as will often be the case, the target enzyme has a low flux control coefficient in the absence of the drug, then activating it is likely to have no perceptible effect, and inhibiting it may have little effect either unless the degree of inhibition is very large, maybe larger than one can realistically expect to achieve. As the aim in any pharmacological project is to produce effects on a whole system, it is essential to have some understanding of the whole system — not just one component of it — if one is to have any hope of

a rational approach to design. At the very least, one needs to have a picture of the the whole pathway in which the drug is intended to act, and one needs measurements of the metabolic importance of any chosen target enzyme in this pathway. Without this, there is little reason to assume that any effects a particular drug may have on a particular enzyme will be translated into perceptible effects on the whole system.

We must distinguish, here, between total inactivation of an enzyme and less drastic effects that an inhibitor may have. If one is fortunate enough to encounter a reagent that totally, specifically and permanently inactivates a particular enzyme, then it is not necessary to have any detailed knowledge of the metabolic pathway in which the enzyme participates to have a reasonable certainty that if one can find a way of delivering the reagent to the target then it will have drastic metabolic consequences. The only obvious exception will be the case of an enzyme whose role is duplicated by another enzyme or pathway that is not inhibited by the reagent in question, though it is hardly likely that one will find a reagent so specific that it inactivates one enzyme without any affect on other isoenzymes. This is not, however, the case most relevant to the *design* of drugs: one may be lucky enough to encounter such a molecule by chance, but the only molecules one can expect to find by design are ones whose activity can be predicted from knowledge of the structure and properties of the target enzyme, and such molecules are unlikely to produce total inactivation.

A second general point that is rarely recognized, though it is crucial, is that competition works both ways: any molecule that can compete with the substrate for binding to the active site of an enzyme is a molecule that the substrate can compete with. What this means in the practical reality of drug design is that unless a competitive inhibitor binds extremely tightly (in which case we are again moving away from design as such) a modest increase in the substrate concentration will normally suffice to overcome its effect. Let us put this into perspective by supposing we are able to deliver a competitive inhibitor to its target at a concentration equal to its inhibition constant, and that the normal concentration of substrate in the absence of the inhibitor is equal to its Michaelis constant. In the usual case of an experiment in the spectrophotometer we should expect addition of the inhibitor in these conditions to decrease the rate from half the limiting rate to one third of the limiting rate, i.e. to decrease the rate by one third, and we might hope that such a decrease would be metabolically useful. This is the wrong calculation, however: in the cell enzymes do

not find themselves in the typical environment of a spectrophotometer cuvette, with no added product, no other enzymes present, a substrate supplied at a defined concentration and able to deliver whatever rate emerges from the Michaelis–Menten equation. On the contrary, they always exist in the company of other enzymes catalysing reactions that involve their substrates and products. Products are usually present, and most enzymes have to transform substrates at rates that are largely determined by the other enzymes around them. Thus they are closer to a defined-rate regime than the defined-concentration regime of the spectrophotometer. The question to ask, therefore, is not how much effect an inhibitor will have on the rate, but how much it will have on the metabolite concentrations. The answer, for the same conditions defined above, and ignoring products, is that doubling the substrate concentration is sufficient to overcome the inhibition entirely. If we do not ignore products then a *smaller* increase in substrate concentration than this will suffice.

Yet unless the substrate is a major metabolite like ATP or is normally present at high concentrations a doubling of its concentration is likely to pass completely unnoticed by the metabolic system in which it exists. It follows that the pharmacological effect we should expect to result from delivering a competitive inhibitor to its target at a reasonable concentration is no effect whatever! To do better than this we must find either a competitive inhibitor that binds so tightly that it is effective at very low concentrations, or we must find one for which the substrate potentiates rather than counteracts the inhibition: in other words any competitive effect that a drug may have is largely irrelevant to its effects i*n vivo;* what is important is any uncompetitive component in the inhibition (Cornish-Bowden, 1986).

Let us now try to apply some of these ideas to *Trypanosoma brucei*. Like some other protozoans, such as *Trypanosoma cruzi,* the organism responsible for Chagas disease in South America, it contains an unusual organelle known as the glycosome in which most of the reactions of glycolysis occur. Unlike these other protozoans, however, it has an extremely specialized metabolism consisting of very little else apart from glycolysis, and in its bloodstream form it is wholly dependent on glucose from its host's blood. In conditions of starvation *Trypanosoma cruzi* can use other energy sources, but *Trypanosoma brucei* cannot, because it lacks not only energy stores but also a functional citric acid cycle. This means that one can obtain a fairly complete idea of its metabolism by studying its glycosomal glycolysis. Moreover, it is made an especially attractive organism for study by the fact that the kinetic properties of its glycolytic enzymes are known in

as much detail as is available for any organism, thanks to the efforts of the group of Opperdoes over a number of years, and by the fact that a detailed computer model of its metabolism has been published (Bakker *et al.*, 1997).

These favourable characteristics led us to study the potential effects of inhibition of different steps on the metabolism of *Trypanosoma brucei* (Eisenthal and Cornish-Bowden, 1998). This metabolism is summarized in Fig. 1. Notice that although the metabolism shown in the figure is essentially glycolysis, it is not quite glycolysis as represented in standard textbooks of biochemistry. Not only do many of the reactions occur in the glycosome, an organelle unknown to most textbooks, but the several reactions involving glycerol are much more prominent than usual. Two other features are not obvious from

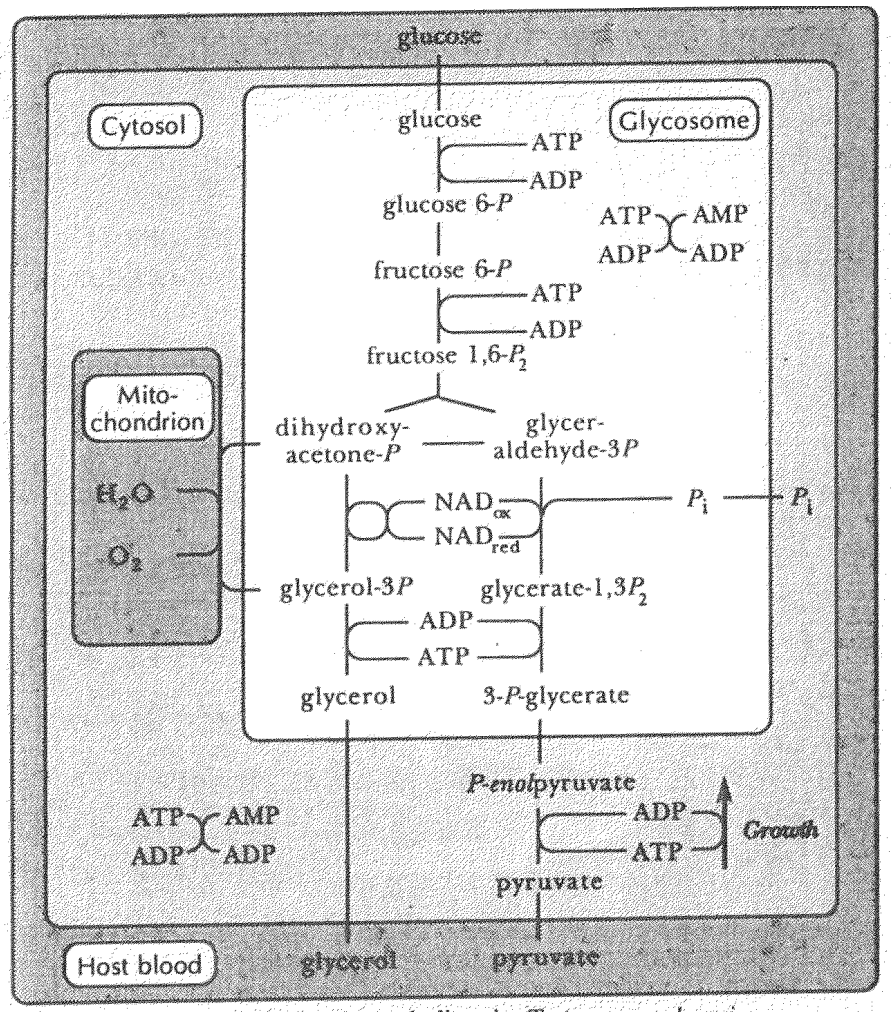

Fig. 1. Outline of metabolism in *Trypanosoma brucei*

the figure but are worth mentioning: first, phosphofructokinase, the archetypal "regulatory enzyme", appears to have none of the usual regulatory properties that have been described in other organisms; second, the vestigial mitochondrion of *Trypanosoma brucei* appears to do little or nothing apart from oxidizing glycerol 3-phosphate.

As the metabolic scheme includes 20 processes (i.e. enzyme-catalysed reactions, blocks of reactions, such as those that connect 3-phosphoglycerate to phospho*enol*pyruvate, and transport steps) there are at first sight 20 potential targets for the action of a drug that might be designed to combat a trypanosomal infection. It turns out, however, that there is only one target where one can expect much chance of success even if one is able to deliver a inhibitor specific to it. To counter any later feeling of obviousness, the reader may like to pause at this point to make a judgement which of the 20 processes illustrated in the figure is the most promising candidate for inhibition, if the objective is to produce drastic metabolic effects.

There are two obvious ways of using an enzyme inhibitor to interfere with the metabolism of a parasite, thereby causing it to die: decreasing an essential flux to a level unable to support life, or increasing the concentration of a metabolite to a toxic level. Neither of these is as straightforward as consideration of isolated enzymes in the spectrophotometer may suggest. Few enzymes in real systems are "rate-limiting" in the sense needed for decreasing the flux to work in the way that one might hope, and computer simulation of glycolysis in *Trypanosoma brucei* suggests that this is no exception. Glucose transport is the only step with a significant amount of flux control, but modelling of the effect of inhibiting it suggested that this would be an unpromising approach in practice unless the degree of inhibition could be made very large: addition of a competitive inhibitor at a concentration equal to its inhibition constant would decrease the transport flux by about 21%, but the growth flux (modelled as the net production of ATP) would decrease by only 15% (Eisenthal and Cornish-Bowden, 1998).

However, in this chapter we are more concerned with the alternative, increasing a metabolite concentration to a toxic level. As discussed already, organisms can overcome competitive effects so easily by slight increases in metabolite concentrations that it is clear that the inhibitor used must be either uncompetitive with respect to the metabolite in question, or there must be a substantial uncompetitive component in the inhibition. This already adds greatly to the difficulty of designing the inhibitor: competitive inhibitors are relatively easy to

design if the reaction mechanism is known, and even without this knowledge there is a good expectation that an unreactive structural analogue of the substrate or product will act as a competitive inhibitor; by contrast, there are few obvious clues as to how to set about designing an uncompetitive inhibitor.

Nonetheless, suppose that combination of the strategies — combinatorial chemistry, screening procedures, antisense oligodeoxynucleotides, structural biology etc. — discussed in *Nature* (1996) allows us to identify a potential uncompetitive inhibitor of one of the 20 processes in glycolysis in the glycosome of *Trypanosoma brucei*. Which process should we choose, and how likely is it that it will work?

Before proceeding further we should note that in any metabolic system some of the metabolite concentrations are free to adjust to any levels in accordance with the activities of the enzymes that produce or consume them. Some, however, are constrained by the stoicheiometry of the network. An obvious example that applies to most steady states in metabolism is the total concentration of adenine nucleotides. In the absence of significant synthesis or degradation of adenine the total of ATP, ADP and AMP must remain constant, and as none of the three concentrations can be negative (a point of elementary chemistry occasionally forgotten by people who study metabolism in the computer) it follows that none of the three can exceed the fixed total. In Fig. 1 there are two such constraints, because there is no transport of adenine into or out of the glycosome, and thus there are separate totals inside the glycosome and in the cytosol.

A third constraint of the same sort applies to NAD and NADH in the glycosome, and this is equally obvious. Clearly we do not require a computer to recognize any of these three constraints, and it is obvious from the outset that any strategy based on driving the NADH concentration to 1 M and above will be doomed to failure.

There is, however, a fourth constraint that is far from obvious: equally constant is the total amount of phosphate in the glycosome that is *not* accounted for by entry of inorganic phosphate and export (in stoicheiometric amounts) of 3-phosphoglycerate. This is rather complicated to describe, and it leads to an even more complicated relationship involving glycerol 3-phosphate, dihydroxyacetone phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, glyceraldehyde 3-phosphate, 1,3-bisphosphoglycerate, ATP and ADP. As two of these, glycerol 3-phosphate and dihydroxyacetone phosphate, exist in the mitochondrion as well as the glycosome, and as it is the total *amount* of phosphate that is conserved, not its con-

centration, the need to take account of the different volumes of the two compartments adds an extra level of complexity to what is already a complicated relationship.

This fourth constraint was recognized by the computer program used by Bakker *et al.* (1997) to study the system, and was found again by Gepasi (Mendes, 1993), the different program that we used (Eisenthal and Cornish-Bowden, 1997). As we had read the earlier paper before undertaking our investigation we can hardly claim this confirmation as independent. However, computer programs are less emotional about questions of priority than people, and the important point is that Gepasi identified the fourth constraint (as well as the first three, of course) without being supplied with any prior information about the number of constraints implied by the stoicheiometry.

This observation establishes the first of our claims, that computer analysis can lead to new information about the structure of a metabolic pathway that is not obvious, even qualitatively, from inspecting it. Nonetheless, the identification of a fourth constraint, one so complicated that it is not easy to describe in words, is not the kind of discovery most biochemists find very exciting. So we need to examine the second half of the claim, that without knowledge of the stoicheiometric constraints (or at least, without knowledge of their consequences) it would be very difficult to predict which process in the pathway would lead to drastic changes in metabolite concentrations if it was inhibited uncompetitively.

The point about the fourth constraint is that once it is taken into account it becomes clear that almost all of the metabolite concentrations in the trypanosome are involved in stoicheiometric constraints, i.e. that they cannot be increased catastrophically. Even glucose, though not involved in any of them, is constrained by the separate fact that as entry of glucose is not an energy-driven process the internal glucose concentration cannot exceed the external concentration. The extremely high glycolytic rate of *Trypanosoma brucei* means that this is a less severe restriction than it would be in most cells, but it is still severe enough to leave little scope for poisoning the organism with excess glucose.

We are left with a mere three out of the apparent total of 20 processes that are realistic candidates for blocking with an uncompetitive inhibitor: glycerol transport, the block of three steps (phosphoglycerate mutase, enolase and phospho*enol*pyruvate transport) represented as a single process converting glycosomal 3-phosphoglycerate to cytosolic phospho*enol*pyruvate, and pyruvate transport. Of these, the block of three steps is ruled out by the fact that it is believed to be close

to equilibrium, and glycerol transport is ruled out by the fact that it carries a such low flux anyway in the aerobic conditions that exist in an infection that it appears to fulfil no necessary function in these conditions; some authors doubt that it occurs at all in the living trypanosome in the aerobic state, and in the model it can be eliminated entirely with little effect on anything else. *(Trypanosoma brucei* also has an anaerobic metabolism, and glycerol transport then becomes essential, as it provides the only way of removing the glycerol 3-phosphate that has to be produced if the NAD consumed in the oxidation of glyceraldehyde 3-phosphate is to be regenerated.)

Finally, therefore, we are left with just one potential candidate for attack with an uncompetitive inhibitor, pyruvate transport. Fortunately this behaves in the model just as one would hope: uncompetitive inhibition of pyruvate transport causes a catastrophic increase in the pyruvate concentration (Eisenthal and Cornish-Bowden, 1998), so it is quite realistic to hope that the trypanosome could be poisoned in this way. It is also fortunate that mammals are less profligate than *Trypanosoma brucei* in their use of glucose: instead of just excreting the products of glycolysis (other than ATP) into the environment they oxidize them further to generate far more ATP than is available from glycolysis alone. This means that one can expect that interfering with the excretion of pyruvate is less likely than inhibiting a glycolytic enzyme to have harmful side-effects in the host.

It remains to be seen whether this will work as well in a patient infected with *Trypanosoma brucei* as it does in a computer model. Nonetheless, it seems clear that computer analysis of the system has provided pointers to possible strategies for treating infection that could not easily have been found otherwise. In particular, it has shown that some metabolic knowledge is essential: searching for new drugs with combinatorial chemistry, structural biology and so forth, but ignoring basic principles of metabolism and enzymology is little more than searching in the dark.

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