those for standard base pairs. Remarkably, pyrene was inserted opposite an abasic template lesion only about fourfold less efficiently than T opposite A. Furthermore, compared with insertion of A opposite the lesion, insertion of pyrene was at least 100-fold more efficient. The relevance of this latter result is that insertion of A opposite an abasic lesion is a common default mechanism, known as the "A rule,"[5] and is used by many polymerases to copy past noncoding lesions that tend to block replication. In earlier studies, Kool and colleagues showed that Klenow fragment incorporated base pairs using difluorotolvene, a non–hydrogen-bonding analog of thymine, at roughly 1/50th the rate of T•A pairs (see, e.g., ref. 2). This is an impressively high incorporation frequency when compared with ~10^{-4} to 10^{-5} incorporation frequencies for C•A mispairs, which are "stabilized" by two hydrogen bonds, in a protonated wobble[6] different from the normal W–C conformation.

Are there potential new research and commercial applications for these and other nonpolar base analogs? In the research arena, it will now be possible to measure the contribution of base stacking to fidelity in the absence of hydrogen bonding. This information can yield potentially new insights into sequence context effects on polymerase fidelity, for example, mutational hot spots, a critically important subject that is barely understood. These base analogs can also be used with a variety of polymerases to investigate active-cleft differences. Since pyrene nucleotide is highly fluorescent, pre–steady-state fluorescence depolarization measurements can be used to analyze constraints on the rotational motion of pyrene in the pol active cleft.

There may also be important practical commercial applications. S.A. Benner and colleagues[7] have used isoC•isoG base pairing in an in vitro coupled transcription–translation assay to encode an unnatural amino acid into a protein. It is conceivable that non–hydrogen-bonding pairs could be also used to expand the genetic code much further. The new pairs may also be used in a SELEX (systematic evolution of ligands by exponential enrichment)-based method to expand the chemical characteristics of DNA to generate new protein-binding ligands (see, e.g., ref. 8). However, this method requires not only that the base analog be copied by the polymerase, but also that extension past the aberrant base pair must occur with reasonably high efficiency. Although extension past pyrene appears highly inefficient, this limitation does not apply to difluorotoluene.

Another application makes use of the property that pyrene stacks strongly and can therefore be used to increase the melting temperature of duplex DNA by placing it at the end, in a dangling position[9]. One can easily envision how an increase in hybridization efficiency can be used in gene chip technology applications designed to capture specific DNA sequences. Finally, Kool and colleagues[10] have used adjacent binding of pyrene-containing oligonucleotides as a color-changing reporter of nucleic acid sequences in solution—pyrene fluoresces blue as a monomer or white-green as an excimer pair. Such probes are sensitive, even to single base differences in target DNA.

Freed from the constraint of needing hydrogen bonding to achieve rapid, high-fidelity DNA synthesis, one may be able to synthesize a wide variety of designer-defined sterically complementary compounds to expand the potential use of DNA in newly minted areas of DNA computation, electrical conductivity, and nanoscale fabrication. Ironically, it may eventually turn out that the "fidelity" of synthesis using non–hydrogen-bonding space-filling substrates can surpass what "hath been wrought" through evolution.

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Metabolic control analysis in biotechnology and medicine

Athel Cornish Bowden

Genomics has opened new possibilities for applications in biotechnology and medicine. For these to be realized, one must not forget that most gene products are proteins, most proteins are enzymes, and most enzymes operate as components of metabolism. It is therefore important to put genetic information in the context of what has long been known about how enzymes behave and how they are regulated in metabolism. Without such a framework, there is little chance of predicting the effects of mutations, deletions, or insertions of genes. The relationship between metabolic control and biotechnology was the unifying theme of a recent NATO Advanced Research Workshop at Visegrád, Hungary*. As one of the organizers of the meeting, I was struck by the closeness of that relationship, even given my inevitable bias and enthusiasm for the field.

Metabolism is strikingly absent from discussions of drug design; for example, it is not even mentioned in a special issue of *Nature*[1] devoted to intelligent drug design. Much the same applies to efforts to increase yields of commercially desirable metabolites: recognition that the process to be modified is metabolic is not enough to avoid the simple-minded idea that once a way has been found to modify the activity of a target enzyme in vivo, the metabolic effects are so obvious that they do not need to be thought about in advance.

Yet it has been known for more than a quarter of a century that the underlying assumption is false: the expected result of a moderate change in the activity of an arbitrarily selected enzyme is that there will be no perceptible change in metabolic flux and no change in phenotype. At one level, of course, this is perfectly familiar: the phenotypes of heterozygotes for recessive genes are normal, or, in biochemical terms, decreasing the activity of an arbitrarily selected enzyme by 50% typically produces no obvious effect.

Metabolic control analysis developed from observations of this kind. Although it remains little known by many active biotechnologists, it may well provide the key to understanding why modern drug design is not noticeably more efficient or successful than it was 30 years ago, and why the impact of genetic manipulation on the production of commercial metabolites has been likewise disappointing. More optimistically, the study of metabolic con-

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trol analysis may provide pointers toward greater success in the future.

Taking cancer as an example of how a better understanding of metabolism may lead to progress, can control analysis shed light on its causes? Can it open the door to better approaches to treatment? Perhaps surprisingly, the answer to both questions may be yes.

The close fit between the continents was obvious to everyone who looked at a world map since accurate maps existed; similarly, the association between aneuploidy and cancer has been obvious to everyone who studied it for more than 100 years. But just as continental drift was not taken seriously by most geophysicists until plausible mechanisms were worked out, most experts in recent decades have regarded aneuploidy as a side effect of cancer, rather than its cause, because there seemed no reason why chromosomal abnormality should result in uncontrolled cell proliferation.

If Peter Duesberg and David Rasnick (University of California, Berkeley) are right, however, aneuploidy is indeed the cause, and metabolic control analysis reveals why. Not only is the association between aneuploidy and cancer so close as to be virtually exact, but the predicted metabolic effect of overexpressing a large and arbitrary set of genes is just the collapse of normal regulation seen in cancer. Altering just one enzyme activity rarely produces much effect, as we have seen, but simultaneous alterations in many activities can overwhelm the normal controls. Down's syndrome illustrates the severe effects that even a minimal degree of aneuploidy can produce—though for people who believe that changing a single enzyme activity can be important this is the wrong example; rather than trying to explain why sufferers are abnormal, they need to explain why, with hundreds of genetic alterations, they are as normal as they are.

So much for the causes of cancer, what about its treatment? In her talk, Marta Cascante (University of Barcelona, Spain) suggested that the enzyme transketolase might play a major role in the control of the nonoxidative pentose-phosphate pathway in cancer cells. This is important not only for exploring the role of transketolase inhibitors as anti-cancer agents, but also for evaluating the current practice of supplementing chemotherapy with thiamine, a cofactor of transketolase, which may do more harm than good.

Metabolic control analysis can also reveal drug targets for use in infectious disease. Figure 1 shows energy metabolism in *Trypanosoma brucei*, the parasite responsible for African sleeping sickness. On the surface, the scheme appears of little use in suggesting likely targets for antiparasitic drugs. But a stoichiometric analysis, without the need for identifying the metabolites and enzymes or even any kinetic information, reveals an almost complete lack of targets for inhibitors that act like the herbicide glyphosate (which does not have a major effect on flux but produces a huge increase in the shikimate concentration).

As Barbara Bakker (University of Delft, Netherlands) discussed, nearly all of the metabolites in the glycosome of the parasite are affected by stoichiometric constraints and so their concentrations cannot increase indefinitely. By combining this approach with the abundant kinetic information available for the enzymes of *T. brucei*, she described how the scheme could be used to construct a computer model that allows the control structure to be deduced.

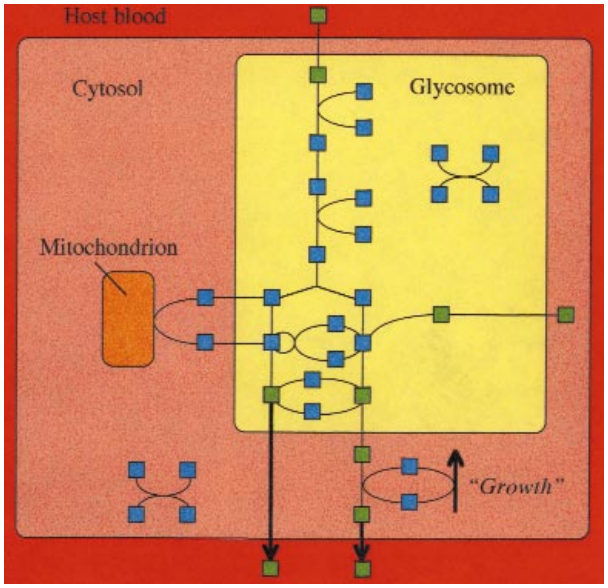

Figure 1. Identifying a target for a drug in *Trypanosoma brucei*. Blue squares represent metabolites whose concentrations are restricted by stoichiometric constraints; green squares represent other metabolites.

The results shed light on likely targets for inhibitors that act in the more familiar way of decreasing the glycolytic flux to a level that no longer supports life. The model suggested target enzymes far from what readers of elementary textbooks might guess. Aldolase, for example, has significant flux control in trypanosomes, but virtually none in the erythrocyte. It is thus a promising target for a drug because, even if it cannot distinguish between the parasite and host enzyme, its metabolic effect will be significant for one and negligible for the other. The more obvious choice of phosphofructokinase proves to be a bad target from any point of view.

Why is the common notion that phosphofructokinase is the control site for glycolysis wrong, and why does overexpressing it or any other supposed rate-limiting enzyme usually fail? According to Jan-Hendrik Hofmeyr (University of Stellenbosch, South Africa), it turns out that the theory of supply and demand works much better in metabolism than it does in economics, and the synthetic fluxes toward most metabolites are regulated by metabolic demand. Increasing the supply has little or no effect because, in the absence of demand, the feedback mechanisms simply switch off the excess capacity.

With this understanding, we can predict where we may expect to see exceptions; in the liver, for example, the primary reason for glycogen synthesis is not to satisfy the liver's need for glycogen, but to prevent hyperglycemia. This is thus a supply-driven pathway, and according to Lorane Agius (University of Newcastle, UK), hexokinase D, the first enzyme in the supply block, proves to have virtually total control—a property that may explain the autosomal dominant inheritance of maturity-onset diabetes of the young.

All living organisms achieve something apparently impossible; they respond to changed conditions with large changes in metabolic flux, but small changes in metabolite concentrations. This could not happen in a pathway regulated by interaction of an effector with a unique regulatory enzyme because the flux control coefficient would be too small and the concentration control coefficient too large.

As David Fell (Brookes University, Oxford, UK) related in his talk, this leads to the idea, now well supported by experimental evidence, that effective regulation requires interaction of signal metabolites with numerous different enzymes. Hans Westerhoff (Free University of Amsterdam, The Netherlands) took this one step further, suggesting that a complete understanding of metabolic regulation is not to be found within metabolism itself, because there are other hierarchical levels of control that affect enzyme concentrations, such as proteolysis and gene expression.

Small changes in substrate, product, or effector concentrations normally produce

only small changes in enzyme activity. According to María Luz Cárdenas (Institut Fédératif Biologie Structurale et Microbiologie, Marseilles), covalent modification cycles between active and inactive forms of enzymes provide a way out of this difficulty. They consume energy, as the conversion reactions need to be irreversible in both directions, so they cannot be used indiscriminately throughout metabolism, but for certain purposes, such as the regulation of glycogen synthesis, they allow a very high degree of sensitivity to signals—so high, indeed, that an important intellectual problem is to understand why the mechanisms that exist in nature are often more complicated than appears necessary just for achieving high sensitivity.

In his talk, James Liao (University of California, Los Angeles) demonstrated that metabolism can indeed be regulated and redirected when engineering objectives are pursued with the aid of control analysis. But as these objectives become more sophisticated, more of what we know of living organisms, especially the complex structures of their cells—far from mere bags of enzymes—will have to be taken into account.

The basic idea that biochemistry is much the same in all organisms has taken our knowledge a tremendous distance, but, as Douglas Kell (University of Wales, Aberystwyth, UK) emphasized in his presentation, real organisms vary widely, not only between species, but also even between individuals in the same "homogeneous" bacterial culture. It will not be possible to ignore the differences forever.

For too long, the central idea in discussions of metabolic regulation has been that everything can be understood in terms of the properties of a few components.

The analysis of the mathematical structure of metabolic pathways and the identification of elementary flux modes could easily, but mistakenly, be regarded as highly academic, with no application to the real world. Talks by Stefan Schuster and Reinhart Heinrich (Humboldt University, Berlin) stressed that it becomes essential if one is to have any hope of making rational assessments in advance of the likely effects of inserting new steps in metabolic pathways or deleting existing ones. Likewise, the evolutionary design of pathways is a guide to how well modified metabolic systems are likely to work.

Theoretical discussions and mathematical models sometimes seem remote from reality, but control analysis is becoming a necessary component in understanding how metabolic systems behave—as was borne out by the talks of Louis Hue, Catholic University of Louvain, Brussels and Paul Srere, Veterans Affairs Medical Center, Dallas, TX—which in turn can be used to modify their behavior for medical or technological ends. For too long, the central idea in discussions of metabolic regulation has been that everything can be understood in terms of the properties of a few components. As Henrik Kacser remarked, "One thing is certain: to understand the whole, you must look at the whole."

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