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Westerhoff, H.V.

DOI

[10.1016/S0167-7799\(00\)88955-6](https://doi.org/10.1016/S0167-7799(00)88955-6)

Publication date

1995

Published in

Trends in Biotechnology

[Link to publication](#)

Citation for published version (APA):

Westerhoff, H. V. (1995). Subtlety in control-metabolic pathway engineering. *Trends in Biotechnology*, 13, 242-244. [https://doi.org/10.1016/S0167-7799\(00\)88955-6](https://doi.org/10.1016/S0167-7799(00)88955-6)

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Glossary

International Standards Organization (ISO) – The international equivalent of CEN. The ISO was founded in 1946 and is based in Geneva. It has played no direct role in the work of TC 233, but works along broadly similar lines to CEN.

ISO 9000 – The general quality standard adopted by the ISO that describes a set of standards aimed at ensuring good, reliable and consistent performance. It is widely used in the manufacturing and service industries.

Good Manufacturing Practice (GMP) – A code of practice covering all aspects of a manufacturing process with the aim of ensuring that products are consistently manufactured to the requisite quality.

FDA Codes of Practice – These are laid down by the US Food and Drug Administration for manufacturing processes, and are of particular importance for getting product approval in the USA.

many companies operate to GMP, ISO 9000 and FDA standards (see Glossary). Inevitably, the new CEN standards impinge on these existing standards, but care is being taken to avoid conflict between them. Where good standards already exist (for example, for safety cabinets), current requirements are being incorporated into the CEN standards. Therefore, the resulting standards are likely to complement existing guidelines, such as the biosafety advice of the UK Advisory Committee on Genetic Modification. The focus on biosafety that drives the CEN standards means

that areas not covered by existing standards are now being developed. Although the standards are not meant to be legally binding in the national sense, conflicts of national interest are bound to arise during the consultation process; the CEN will need to sift through the technical and political nature of these comments.

International aspects

The new standards have the potential to benefit European biotechnology by giving it a lead in health and safety, environmental and consumer protection; they offer the

advantage of a common approach and terminology for standardization. International companies may well prefer to use a European, rather than a nationally based, standard. Indeed, there is a general feeling that companies would now like to see written standards replace informal discussion as a basis for doing business.

Furthermore, there has been little activity in this area in Japan, and the long-awaited paper on US standardization has yet to appear. However, there are indications that the US biotechnology sector feels that the lack of standardization makes it hard to get products onto the market, so it may yet initiate its own programme. Therefore, there is a real opportunity for Europe to take a lead. If industry gets fully involved, the standards could be of high enough quality to be adopted by the ISO.

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Susan Aldridge

is a freelance science writer.

forum

Meeting report

Subtlety in control – metabolic pathway engineering

Once, it all seemed so simple: the next generation of microbial bioreactors would be produced by modulating the rate-limiting enzyme for each desired product. However, when rate-limiting enzymes were overexpressed, the pathway flux was hardly affected. In addition, the very basis for expecting there to be single rate-limiting steps has become questionable. Cells control their metabolic pathways much more subtly than anticipated. Therefore, a new generation of microbial strains and a more sophisticated approach to metabolism were required. One such approach, Metabolic Control Analysis (MCA), was first developed by the

late Henrik Kacser and by Jim Burns in Edinburgh¹, and by Sam and Tom Rapoport, and Reinhart Heinrich in Berlin². A recent conference* highlighted how approaches such as MCA have become essential for the analysis of metabolic control and for the rational engineering of metabolic pathways.

Exit the foremost example of a rate-limiting step: phosphofructokinase

At the meeting, it was obvious that MCA has reached adulthood¹: its principles and merits were no longer subject to discussion, no one looked seriously for the single rate-limiting

step, and the terms flux-control coefficient[†] and elasticity coefficient[‡] needed no introduction. In addition, the prime example of the rate-limiting step ceased to exist as such. For years, phosphofructokinase (PFK-1,6) had been cited as the rate-limiting, pace-making, or key enzyme for steady-state glycolytic flux, and for glycolytic oscillations.

*The First European Conference on 'Control of Metabolic Flux: Metabolic Pathway Engineering in Yeasts' was organized by Alistair Brown, Juana-Maria Gancedo and Peter Niederberger, and was held in Granada, Spain, 7–12 April 1995. The conference was funded under the EUROCONFERENCES initiative of the European Union.

† Flux-control coefficient: the extent to which an enzyme controls a steady-state flux; usually a value between zero and one.

‡ Elasticity coefficient: the extent to which the rate of reaction of an enzyme responds to changes in the concentration of a substrate, product, or effector (its kinetic order).

When Zimmermann's group demonstrated that the overexpression of glycolytic enzymes, such as PFK-1,6, did not increase glycolytic flux³, it was suggested that this was due to a shift of control away from the over-expressed enzyme. Alistair Brown (University of Aberdeen, Aberdeen, UK) has demonstrated that a reduction in the concentration of PFK-1,6 has no apparent effect on glycolytic flux either. Clearly, it does not matter whether the concentration of PFK-1,6 is increased or reduced: the control coefficient of PFK-1,6 is close to zero, and the enzyme is far from being rate limiting. Hans Westerhoff (ECIS and Free University, Amsterdam, The Netherlands) showed that the frequency of glycolytic oscillations decreases significantly with decreasing concentrations of extracellular mannose. A comparison of this with the change in the mannose-transport rate in mannose-transport assays suggested that the mannose transporter controlled the frequency of the oscillations for some 70%. As the sum of all control coefficients with respect to frequency must equal 100% (Refs 1,2), it is unlikely that PFK-1,6 exerts much control on the frequency; its control cannot exceed 30%. Exit PFK-1,6 as the rate-limiting step.

Glucose transport

Does sugar transport also control steady flux? Michael Ciriacy (Heinrich-Heine University, Düsseldorf, Germany) reported that 14 *HXT* genes in *Saccharomyces cerevisiae* are homologous to monosaccharide-transporter genes. When *HXT1*–*HXT7* were deleted, glucose uptake and growth on glucose were virtually abolished. The transport $V_{\max}$ of the strains containing only one of the *HXT1*–*HXT7* genes was always lower than that of the wild type, and the growth rate decreased in parallel with $V_{\max}$. However, overexpressing the *HXT1* gene with the *ADH1* promoter did not cause an increase in the growth rate beyond that of the wild type, and the growth rate remained lower than the maximum transport rate. It appears that the glucose transporter coefficient of control over growth rate and glycolysis may not be very high.

Strains containing only the *HXT1*, *HXT3* or *HXT4*⁺ genes grew on high concentrations of glucose and exhibited low-affinity transport; by contrast, strains containing only *HXT2*, *HXT6* or *HXT7*⁺ grew on

low concentrations of glucose and exhibited high-affinity transport – affinities were independent of whether the carriers were expressed at low or high glucose concentrations. This suggests that changes in the affinity of glucose transport with the extracellular glucose concentration are due to differential expression of carrier genes, rather than to modulation of the affinity of a single carrier⁴. Indeed, *HXT1* was expressed at high, but not low, glucose concentrations; this was not the case for *HXT7*.

Elusive control, control hierarchies and EUROFAN

The recurring theme at the meeting was the observation that few enzymes live up to the expected high control coefficients. Juana-Maria Gancedo (CSIC, Madrid, Spain) reported that expression of phosphoenolpyruvate (PEP) carboxykinase, or fructose-1,6-bisphosphatase (FbPase-1,6) in yeast growing on glucose did not reduce cellular ATP levels. Jean François (Centre de Bio-ingénierie Gilbert Durand, Toulouse, France) showed that large changes in the amount of enzyme that catalyses the reversible formation of UDP-glucose did not affect the growth rate of *S. cerevisiae* on glucose, and Westerhoff showed that the H⁺-ATPase did not control growth rate in *Escherichia coli*.

Living cells appear to activate subtle homeostatic mechanisms when attempts are made to perturb cell physiology. These manifest themselves in different ways: Gancedo observed an increase in respiration; Terry Cooper (University of Tennessee, Knoxville, TN, USA) showed that deletion of the *ure2* gene, encoding a protein proximal to the signal for nitrogen catabolite repression (NCR), caused responses varying greatly in intensity among NCR-sensitive genes. In many cases, the absence of control appears to be due to a control hierarchy that is outside the metabolic pathway. These include the observations that: overexpression of PFK-1,6 causes a decrease in PFK-2,6 concentration, as well as a decrease in the concentration of fructose 2,6-bisphosphate, which is the product of the PFK-2,6 reaction and an activator of PFK-1,6 (Kevin Brindle, University of Cambridge, Cambridge, UK); the concentration of b-cytochromes increases when the *E. coli* H⁺-ATPase is down-modulated (Westerhoff);

the expression of *HXT1* is suppressed by *HXT7* (Ciriacy); different glycolytic genes are induced by different changes in the levels of glycolytic metabolites (Eckhard Boles, Technische Hochschule, Darmstadt, Germany); and the glucose inducibility of the single low-affinity glucose transporter of *Kluyveromyces lactis* is impaired in mutants deficient in phosphoglucoisomerase, hexokinase and PFK-1,6 (Wésolowski-Louvel, Institut Curie, Paris, France).

Paradoxically, it was observed that regulatory circuits do not appear to control steady-state flux either. Jürgen Heinisch (Heinrich-Heine University) reported that removal of the fructose-2,6-bisphosphate binding site from PFK-1,6 did not affect the growth of *S. cerevisiae*, neither did replacing the normal promoters of the PFK-1,6 and pyruvate kinase genes with the phosphoglycerate kinase promoter (Brown). The lack of effect on steady-state flux, observed when removing regulatability at constant activity, is understandable. To highlight the control exerted by regulatory circuits, it is necessary to study response times or sustained oscillations (periods of one minute – Peter Richard, ECIS, Amsterdam, The Netherlands; and periods of 40 minutes – Marc Keulers, NIBHT, Ibaraki, Japan). The synchronization of the oscillations between individual cells is a newly discovered control point, with acetaldehyde serving as the intercellular signalling (Richard and Westerhoff).

When describing the EUROpean Functional Analysis Network that he is setting up, Steve Oliver (UMIST, Manchester, UK) discussed the subtlety of control that leads to small control coefficients. Screening and homology searches have revealed that 45% of the yeast open reading frames (ORFs) have no phenotype and no known function. Oliver proposes to deploy modular (top-down) MCA to locate where the gene product is active, and then to assign that gene to a node of research groups specializing in that aspect of cell function. The tools that will be used include: MCA; screening under competition (shown to be effective by Gancedo for her deregulated FbPase strains) and transient conditions; and screening in new backgrounds with shifted control. Whereas the 55% of known genes have often confirmed what is already known, Oliver's proposed analysis

of the 45% genes of unknown function is likely to reveal completely new regulatory mechanisms and principles.

Methods and concepts: MCA, flux analysis, bottlenecks and overflow

In view of the subtleties observed in metabolic control, the development of more sophisticated experimental and theoretical tools seems appropriate. Rankin Small (University of Edinburgh, Edinburgh, UK) described two recent advances that have been contributed by Henrik Kacser's group. The so-called deviation index predicts the effects of large changes in enzyme activities with remarkable accuracy. By simultaneously modulating the activity of a number of enzymes in a predictable manner, it should be possible to obtain significant changes in flux. Jay Bailey (ETH, Zürich, Switzerland) introduced non-linear models; he has calculated a single, optimum design for his model of yeast glycolysis, out of the 2^{15} designs that are possible when changes in enzyme activities are restricted to a factor of two, and elasticities are either present or absent.

Flux analysis complements MCA: it can be used to calculate individual reaction rates using metabolic maps and measured input and output flows. Only five fluxes have had to be measured in order to calculate the rates of 58 reactions in *S. cerevisiae* (Ulrik Schulze, Technical University of Denmark, Lyngby, Denmark). When he performed flux analysis on *E. coli* growing on various carbon sources, Harry Holms (Bioflux, Glasgow, UK) found that acetate secretion acts as a safety valve for occasions when the carbon metabolite pools receive an excessive supply of substrate. Peter Niederberger (Nestec, Lausanne, Switzerland) is implementing Accelerated chemostat methodology to reduce overflow into the pyruvate-bypass C_2 compounds that occur at high glucose concentrations. Hans van Dijken (Kluyver Laboratory, Delft, The Netherlands) identified a bypass of pyruvate dehydrogenase as an overflow valve for *S. cerevisiae*: mutants deficient in pyruvate dehydrogenase can grow on low glucose concentrations at a reduced growth yield (the bypass has a reduced ATP yield). However, mutants lacking pyruvate decarboxylase cannot grow on glucose alone,

but the inclusion of small amounts of acetate will support growth. Therefore, van Dijken proposed that mitochondrial and cytosolic acetyl-CoA cannot complement each other. When the ratio of ethanol to glucose was increased, five distinct metabolic regimes appeared. The transitions were accompanied by an induction of the relevant enzymes, i.e. the glyoxylate cycle, PEP carboxykinase and FbPase-1,6, and some sort of hierarchical control seems to be involved.

No channelling

The shikimate pathway seemed to be a prime candidate for metabolite channelling, due to the fact that five consecutive enzyme activities reside on a single polypeptide chain (the AROM protein). However, Alastair Hawkins (University of Newcastle, Newcastle, UK) showed that overexpression of the enzymes in the quinate pathway, which catalyse the same reactions that occur in the shikimate pathway, diminishes the output of the shikimate pathway, suggesting that no channelling occurs.

Metabolic pathway engineering

The interest in improving microbial productivity was also reflected by the active participation of industry (companies such as Nestlé, Unilever, GIST-Brocades, Carlsberg, Bioflux, Alko, Primalko took part, and Quest even organized its own workshop). MCA was linked to a variety of different topics, including: differences between industrial and academic strains; the importance of autolysis; time-programmable accumulation of product (Hein Stam, Quest, Naarden, The Netherlands); 5'IMP, 5'GMP, acetaldehyde and taste (Kees van der Beek, GIST-Brocades, Delft, The Netherlands); and sulphite as anti-oxidant in beer brewing (Claes Gjermansen, Carlsberg, Denmark). Genetic engineering of an enzyme into yeast to remove diacetyl made it possible to shorten the lagering time of beer. Isabel Calderón and Farfán (University of Seville, Seville, Spain) reported that transformation of *S. cerevisiae* with a plasmid encoding aspartate kinase insensitive to feedback inhibition by threonine could be used to produce a 15-fold increase in threonine, but with reduced cell growth. Matti Korhola (Primalco, Helsinki, Finland) reported various ways of expressing α -galactosidase in

yeast, one of which led to an increase in ethanol yield. Merja Penttilä (Helsinki, Finland) and Nina Meinander (Lund, Sweden) demonstrated that *S. cerevisiae* could be transformed with xylose reductase from *Pichia*, so that it was able to synthesize xylitol from xylose. The yield was improved if limited oxygenation was used, and this reduced the production of acetate from the glucose co-substrate. By transforming with xylitol dehydrogenase as well, *S. cerevisiae* was able to produce ethanol from xylose, while requiring less glucose. Barre and co-workers (INRA, Montpellier, France) manipulated the redox metabolism of *S. cerevisiae* by transforming with lactate dehydrogenase from *Lactobacillus casei*. They found that 40% of the flux could be diverted from ethanol to lactate.

Metabolic pathway engineering is now using conceptual and experimental tools that begin to match the complexity of their object: the living cell.

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Hans V. Westerhoff

Mathematical Biochemistry, University of Amsterdam and Microbial Physiology, Faculty of Biology, Free University, De Boelelaan 1087, NL-1081 HV, Amsterdam, The Netherlands.

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