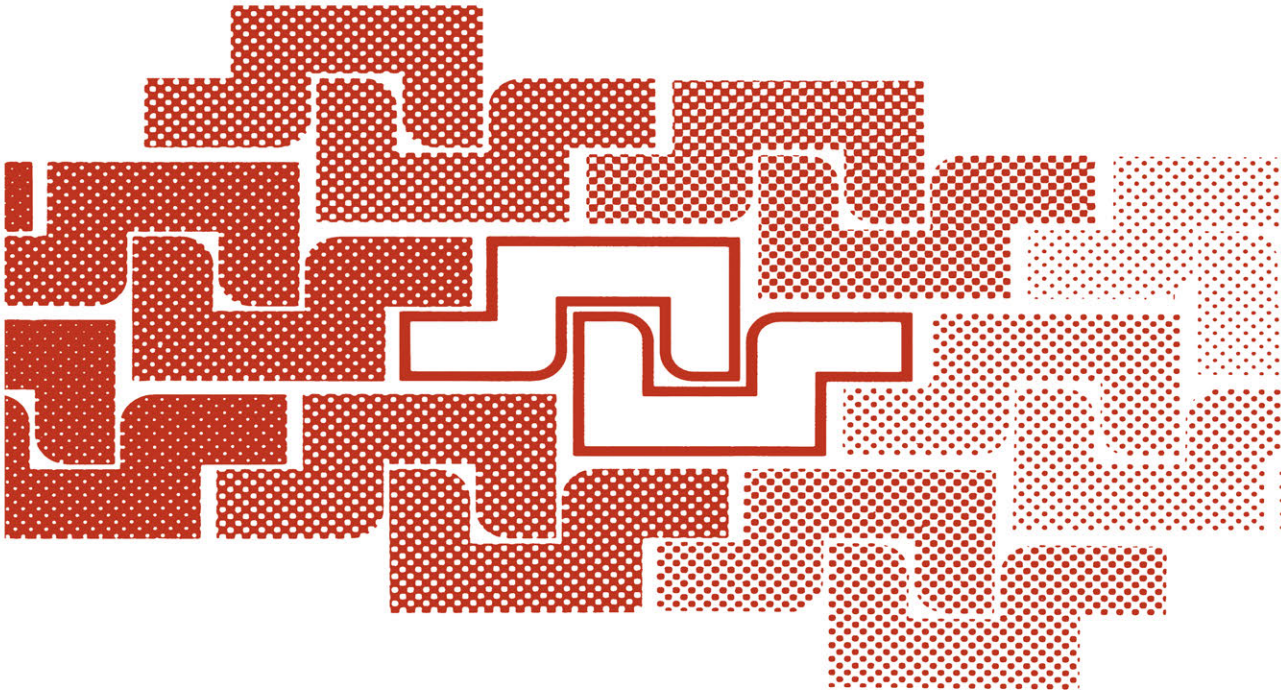


Control of Metabolic Processes

Edited by

Athel Cornish-Bowden and

María Luz Cárdenas

NATO ASI Series

Series A: Life Sciences Vol. 190

Control of Metabolic Processes

NATO ASI Series

Advanced Science Institutes Series

A series presenting the results of activities sponsored by the NATO Science Committee, which aims at the dissemination of advanced scientific and technological knowledge, with a view to strengthening links between scientific communities.

The series is published by an international board of publishers in conjunction with the NATO Scientific Affairs Division

A	**Life Sciences**	Plenum Publishing Corporation
B	**Physics**	New York and London
C	**Mathematical and Physical Sciences**	Kluwer Academic Publishers
D	**Behavioral and Social Sciences**	Dordrecht, Boston, and London
E	**Applied Sciences**	
F	**Computer and Systems Sciences**	Springer-Verlag
G	**Ecological Sciences**	Berlin, Heidelberg, New York, London,
H	**Cell Biology**	Paris, and Tokyo

Recent Volumes in this Series

Volume 184—Bone Regulatory Factors: Morphology, Biochemistry, Physiology, and Pharmacology
edited by Antonio Pecile and Benedetto de Bernard

Volume 185—Modern Concepts in *Penicillium* and *Aspergillus* Classification
edited by Robert A. Samson and John I. Pitt

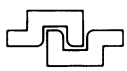
Volume 186—Plant Aging: Basic and Applied Approaches
edited by Roberto Rodríguez, R. Sánchez Tamés, and D. J. Durzan

Volume 187—Recent Advances in the Development and Germination of Seeds
edited by Raymond B. Taylorson

Volume 188—Evolution of the First Nervous Systems
edited by Peter A. V. Anderson

Volume 189—Free Radicals, Lipoproteins, and Membrane Lipids
edited by A. Crastes de Paulet, L. Douste-Blazy, and R. Paoletti

Volume 190—Control of Metabolic Processes
edited by Athel Cornish-Bowden and María Luz Cárdenas



Series A: Life Sciences

Control of Metabolic Processes

Edited by

Athel Cornish-Bowden and
María Luz Cárdenas

Center for Biochemistry and Molecular Biology—CNRS
Marseille, France

Springer Science+Business Media, LLC

Proceedings of a NATO Advanced Research Workshop
on Control of Metabolic Processes,
held April 9-15, 1989,
in Il Ciocco (Lucca), Italy

Library of Congress Cataloging-in-Publication Data

NATO Advanced Research Workshop on Control of Metabolic Processes
(1989 : Lucca, Italy)
Control of metabolic processes / edited by Athel Cornish-Bowden
and María Luz Cárdenas.
p. cm. -- (NATO ASI series. Series A, Life sciences ; v.
190)
"Published in cooperation with NATO Scientific Affairs Division."
"Proceedings of a NATO Advanced Research Workshop on Control of
Metabolic Processes, held April 9-15, 1989 in Il Ciocco, Lucca,
Italy"--T.p. verso.
Includes bibliographical references.
ISBN 978-1-4757-9858-6 ISBN 978-1-4757-9856-2 (eBook)
DOI 10.1007/978-1-4757-9856-2
1. Metabolism--Regulation--Mathemtical models--Congresses.
2. Control theory--Congresses. I. Cornish-Bowden, Athel.
II. Cárdenas, María Luz. III. North Atlantic Treaty Organization.
Scientific Affairs Division. IV. Title. V. Series.
[DNLM: 1. Biochemistry--congresses. 2. Metabolism--congressses.
3. Molecular Biology--congresses. QU 120 N279c 1989]
QP171.N38 1989
574.1'33--dc20
DNLM/DLC
for Library of Congress 90-7160
CIP

Originally published by Plenum Press, New York in 1990
Softcover reprint of the hardcover 1st edition 1990

To Isadora, the youngest participant in the workshop

Preface

THIS BOOK collects together papers given at a NATO Advanced Research Workshop held at Il Ciocco (Lucca), Italy, from the 9th to the 15th April, 1989. It sets out to present the current state of understanding of the principles governing the way fluxes and concentrations are maintained and controlled in metabolic systems. Although this is a topic that has held the interest of biochemists for many years, it is only quite recently that the methods of analysing the kinetics of multi-enzyme pathways developed over the past two decades have come to be widely discussed or applied experimentally. Many biochemists remain sceptical that the new methods offer a real advance (except in complexity) over the landmark discoveries of the 1950s and 1960s relating to inhibition of enzymes at branch-points by the end products of metabolic pathways, and the interpretation of allosteric effects and cooperativity.

Even those who have become convinced that the classical ideas provide only the starting point for understanding metabolic control have been by no means unanimous in their assessment of the direction in which one should advance. In this book we have tried to include all of the current points of view, including the view that the classical theories tell us all that we need to know. We have not seen it as our role as editors to paper over the cracks that exist and to pretend that we can speak to the world with one voice. Nonetheless, at the Workshop that this book records we did try to resolve some of the controversies that were apparent, for example, in the pages of *Trends in Biochemical Sciences* in 1987, and we hope that some progress towards such resolution may be evident in the book.

The Prologue is based on a paper written by Daniel Atkinson without any intention of publication but circulated before the Workshop in the hope of stimulating discussion and focussing the attention of participants on some issues that he believed needed to be addressed. It proved highly successful in doing this, and stimulated a number of additional discussion papers, all of which are also included in the Prologue. We emphasize that this part of the book was written before any of the other chapters and that it has not been edited to take account of anything that may have been written later.

The next part of the book is concerned with general aspects of metabolic control analysis, including discussion of historical and philosophical aspects as well as description of the somewhat different approaches that have developed in Europe and the USA, which cannot yet be considered to have reached a synthesis. This is followed by several chapters dealing with the mathematical basis of control analysis.

Enzymes possessing two forms with different catalytic activity that can be interconverted by covalent modification reactions form a special category of enzymes that need to be studied in relation to metabolic control. The number of experimental examples of these continues to grow rapidly, and we include several chapters discussing their different aspects.

These are followed by some chapters dealing with methods that have been developed for

applying the ideas of control analysis to experimental systems. These methods will, we hope, help to dispel the idea that metabolic control analysis is an abstract subject with little relationship to "real" biochemistry. Control-pattern analysis, discussed in the last chapter of this section, may perhaps in time come to provide the same degree of intuitive understanding of metabolic control that the method of King and Altman has given to enzyme mechanisms.

Direct "channelling" of intermediates from one enzyme to the next in a pathway, other kinds of interactions between enzymes, failure of rates to be strictly proportional to enzyme concentrations, and the fact that real systems do not always operate in the steady state, are all complications that need to be considered before any simple theory of metabolic control can be accepted as providing the whole story. To some degree these complications overlap; in other respects they are quite distinct: we have found it convenient to group together the chapters discussing these various ways in which nature makes real systems more complicated than one might have hoped.

Although in most of these earlier chapters there are sufficient references to experimental systems to show that metabolic control analysis is more than a preserve of pure theory isolated from the real world, the relation between metabolic control analysis and "wet" biochemistry becomes more evident in the final group of chapters in the book. Here it will be seen that the ideas of control analysis are now being applied to all of the classical problems that have engaged the attention of biochemists — erythrocyte metabolism, photosynthesis, amino acid metabolism, and so on. Even gene expression is included, a topic that many of us might have thought too difficult to be yet accommodated in a mathematical treatment of metabolic control. The range of applications will certainly increase in the years to come, and we suspect that it will eventually be thought odd that biochemists could ever have thought that these problems could be addressed without the aid of control analysis.

Readers who see any merit in the lay-out of this book may like to know that it was printed in its entirety from camera-ready copy prepared on an Apple LaserWriter II NT driven by an Apple Macintosh Plus computer: the text was edited using WriteNow for Macintosh (version 2.0, T/Maker Company, Mountain View), and the mathematical expressions were laid out with Expressionist (version 2.0, Allan Bonadio Associates, San Francisco).

We are very grateful to the Scientific Affairs Division of NATO for the grant that made possible the Workshop on which this book is based, and to Dr Craig Sinclair, the Director of the Advanced Research Workshop programme, for his help and encouragement in the organization. Additional financial support provided by various other bodies was also much appreciated, as it enabled the participation of several scientists who would otherwise have been unable to attend the Workshop. In this connection, we thank Dr Paolo Fasella, for a grant from the Commission of the European Communities; Dr Minor S. Coon, for travel grants from the International Union of Biochemistry and the American Society for Biochemistry and Molecular Biology; and Dr Christopher I. Pogson, for a grant from the Wellcome Foundation Ltd.

Many participants in the Workshop have commented to us afterwards how much they enjoyed the time that they spent at Il Ciocco. A major part of the credit for this must go to Mr Bruno Giannasi and his staff, who went far beyond mere obligation in ensuring that everyone was well provided for. We thank them most sincerely. If only we could have had as much success in planning the weather on the half-day excursion ...

We are grateful to Jacques Ricard for suggesting the Workshop in the first place, and to him and the other the members of the Organizing Committee — Albert Goldbeter, Tamás Keleti and Hans Westerhoff — for many useful suggestions and other invaluable help, and to Brigitte Gontero for all of her assistance as Meeting Secretary — before, during and after the Workshop. We also thank the many authors who delivered their text in machine-readable form, thereby reducing the amount of retyping that we had to do. For the chapters that did need to be retyped, we thank Mme Monique Payan for her highly professional work.

We record with great sorrow that Tamás Keleti died suddenly during the period in which the book was in preparation. Many will remember him for his tireless efforts on behalf of enzymology in Hungary; he will be sadly missed, not only by his colleagues there, but also by many enzymologists around the world, and especially by our daughter Isadora.

Athel Cornish-Bowden
María Luz Cárdenas

Contents

Prologue

D. E. ATKINSON
What should a Theory of Metabolic Control offer to the Experimenter? 3

I. General Aspects of Control Theory

A. CORNISH-BOWDEN
1. The Nature and Role of Theory in Metabolic Control 31

J. HIGGINS
2. History and Original Thoughts on the Control Theoretic Approach 41

J. W. PORTEOUS
3. Control Analysis: a Theory that Works 51

M. A. SAVAGEAU
4. Biochemical Systems Theory: Alternative Views of Metabolic Control 69

E. O. VOIT
5. Comparison of Accuracy of Alternative Models for Biochemical Pathways 89

A. K. GROEN AND H. V. WESTERHOFF
6. Modern Control Theories: a Consumers' Test 101

II. Mathematical Aspects of Control Analysis

C. REDER
7. The Structural Approach to Metabolic Control Analysis: I. Theoretical Aspects ... 121

J.-P. MAZAT, C. REDER AND T. LETELLIER
8. The Structural Approach to Metabolic Control Analysis: II. Geometrical Aspects... 129

D. A. FELL, H. M. SAURO AND J. R. SMALL
9. Control Coefficients and the Matrix Method 139

M. CASCANTE, R. FRANCO AND E. I. CANELA
10. Performance Indices in Metabolic Systems: A Criterion for Evaluating Effectiveness in Metabolic Regulation 149

E. I. CANELA, M. CASCANTE AND R. FRANCO
11. Practical Determination of Control Coefficients in Metabolic Pathways 157

III. Interconvertible Enzymes in Metabolic Control

A. GOLDBETER AND D. E. KOSHLAND, JR.
12. Zero-Order Ultrasensitivity in Interconvertible Enzyme Systems ... 173

P. B. CHOCK, S. G. RHEE AND E. R. STADTMAN
13. Metabolic Control by the Cyclic Cascade Mechanism: a Study of *E. coli* Glutamine Synthetase ... 183

M. L. CÁRDENAS AND A. CORNISH-BOWDEN
14. Properties Needed for the Enzymes of an Interconvertible Cascade to Generate a Highly Sensitive Response ... 195

R. D. EDSTROM, M. H. MEINKE, M. E. GURNACK, D. M. STEINHORN, X. YANG, R. YANG AND D. F. EVANS
15. Regulation of Muscle Glycogenolysis ... 209

IV. Methods of Applying Control Analysis to Real Systems

P. MENDES, J. MONIZ BARRETO, A. VAZ GOMES AND A. PONCES FREIRE
16. Methodology for Simulation of Metabolic Pathways and Calculation of Control Coefficients ... 221

H. M. SAURO
17. Regulatory Responses and Control Analysis: Assessment of the Relative Importance of Internal Effectors ... 225

E. MELÉNDEZ-HEVIA AND N. V. TORRES
18. Determination of Control Coefficients by Shortening and Enzyme Titration of Metabolic Pathways ... 231

J.-H. S. HOFMEYR
19. Control-Pattern Analysis of Metabolic Systems ... 239

V. Channelling of Intermediates and the Time Domain

H. KACSER, H. M. SAURO AND L. ACERENZA
20. Control Analysis of Systems with Enzyme-Enzyme Interactions ... 251

T. KELETI
21. Coupled Reactions and Channelling: their Role in the Control of Metabolism ... 259

J. OVÁDI
22. Channelling and Channel Efficiency: Theory and Analytical Implications ... 271

J. S. EASTERBY
23. Temporal Analysis of the Transition between Steady States ... 281

J. RICARD, J. BUC, N. KELLERSHOHN AND J.-M. SOULIÉ
24. Sensing of Chemical Signals by Enzymes ... 291

L. ACERENZA
25. Temporal Aspects of the Control of Metabolic Processes 297

M. MARKUS AND B. HESS
26. Control of Metabolic Oscillations: Unpredictability, Critical Slowing Down, Optimal Stability and Hysteresis 303

VI. Experimental Examples

B. E. WRIGHT AND K. R. ALBE
27. A New Method for Estimating Enzyme Activity and Control Coefficients *in vivo* 317

R. HEINRICH
28. Metabolic Control Analysis: Principles and Application to the Erythrocyte 329

M. N. BERRY, R. B. GREGORY, A. R. GRIVELL, D. C. HENLY, J. W. PHILLIPS, P. G. WALLACE AND G. R. WELCH
29. Constraints in the Application of Control Analysis to the Study of Metabolism in Hepatocytes 343

C. GIERSCH, D. LÄMMEL AND K. STEFFEN
30. Application of Metabolic Control Analysis to Photosynthesis: the Problem of Getting Data for an Impressive Algorithm 351

M. STITT
31. Application of Control Analysis to Photosynthetic Sucrose Synthesis 363

R. G. KNOWLES, C. I. POGSON AND M. SALTER
32. Application of Control Analysis to the Study of Amino Acid Metabolism 377

G. K. SMITH, R. G. KNOWLES, C. I. POGSON, M. SALTER, M. HANLON AND R. MULLIN
33. Flux Control Coefficients of Glycinamide Ribonucleotide Transformylase for *de novo* Purine Biosynthesis 385

A. M. DEAN
34. Molecular Adaptation in the Lactose Operon 389

H. V. WESTERHOFF, J. G. KOSTER, M. VAN WORKUM AND K. E. RUDD
35. On the Control of Gene Expression 399

D. E. ATKINSON
36. An Experimentalist's View of Control Analysis 413

Appendix A: Epilogue (H. KACSER) 429

Appendix B: Computer Programs for Modelling Metabolic Systems 433

Contributors 437

Index 441

PROLOGUE

Prologue

What Should a Theory of Metabolic Control Offer to the Experimenter?

DANIEL E. ATKINSON

IN HIS PREPARATIONS for this symposium, Athel Cornish-Bowden mentioned a desire to present control theory to experimentalists in such a way as to persuade them that their subject could advance more rapidly with more attention to theoretical ideas. He is also inviting some experimentalists to indicate what they think a useful theory should offer, and has asked me to attempt to assess in the final chapter the extent to which the others have offered experimentalists a workable approach to metabolic control.

I applaud this attempt to bring theorists and experimentalists together not only physically but intellectually. However, I remember all too well previous meetings of this kind that I have attended, in which theorists and experimentalists talked past each other with little or no effective interaction. I am concerned that there might not be much for me to say at the end except to deplore the fact that we had once again done so.

In an attempt to avoid finding myself in that unhappy situation, I am setting forth here some of the points that experimentalists wish theorists would consider. I hope that our chances of meaningful interaction will be enhanced if some participants consider these points in advance.

Firstly, and encompassing many of the other points, what experimentalists desire from theorists is relevance. Many model builders appear to believe that control theory is a discrete area of importance in itself, and that it is unnecessary, if not intellectually demeaning, to take the features of actual systems into account in the design of the models. However, even if an autonomous body of concepts does exist, those concepts are of no use to people dealing with an actual experimental or engineering system until they are adapted to relate to specific features of the system. A steam engine, an internal-combustion piston engine, a Wankel rotary internal combustion engine, a gas or water turbine, and an electric motor all generate power and transmit it by way of a rotating shaft. There may be some rarified level of control theory that applies to all of them. But a theory that ignores the existence of valves, an

Daniel E. Atkinson ● Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

ignition system, and mechanisms for introduction of fuel into the cylinders will not be of any practical value to a design engineer who works on piston engines. Similarly, models that ignore the properties of enzymes cannot be helpful to experimental biochemists.

Enzymes are saturable. Their effective kinetic orders change with the degree of saturation. Most enzymes are non-regulatory in the usual sense, and their behaviour is adequately described by the familiar Michaelis equation. In such cases, the momentary kinetic order equals 1 - v/V_{max}. Regulatory enzymes typically have higher, but still variable, kinetic orders. Some of the more mathematically-oriented models ignore the fact that all real metabolic reactions are catalysed by enzymes, and assume reactions of invariant kinetic order one. Since regulation is necessarily a kinetic phenomenon, models that begin with incorrect assumptions as to the kinetic nature of the reactions cannot be useful.

Consideration of the patterns of metabolic conversions seems to indicate clearly that metabolic control is effected in large part by changes in the properties of enzymes that compete for substrates at branchpoints. On the basis of known metabolic patterns and the behaviour of specific enzymes in kinetic studies *in vitro,* it seems to be well established that the outcomes of such competitions at branchpoints are regulated by changes in the affinities of the enzymes for the branchpoint metabolite. No model that does not incorporate those aspects of real metabolic systems is likely to have sufficient relevance to be potentially useful to experimentalists or to contribute to understanding of metabolic control.

Designers of mathematical models tend to aim for generality. Some describe their models as capable of dealing with all possible situations, and it is even claimed by some workers that they begin from first principles. Such aims are illusory and unattainable, and they may lead model designers away from concern with real properties of real systems.

It has been clear for 130 years, since publication of *The Origin of Species,* that there are no general principles, of the type sought by some model builders, in biology. The properties of organisms have been determined historically by selection of beneficial changes from among the vast range of choices made available by random mutation. It is totally and unequivocally impossible to predict the consequences of a long evolutionary history from first principles, or indeed from any mathematical, physical, or chemical principles whatever. Chemical and physical considerations constrain the possibilities — metabolic processes, like all others, must entail a decrease in Gibbs free energy if they occur at constant temperature and pressure, for example — but they cannot lead to specific predictions. The strange sequence of reactions by which glucose is oxidized to carbon dioxide in typical living cells, by way of phosphorylated intermediates and a series of di- and tricarboxylic acids, is a consequence of evolutionary history and could not, even in principle, be predicted. The same considerations apply to the use of the ATP-ADP-AMP pool as the primary transducer of metabolic energy, and to thousands of other features of metabolism. Mechanisms of correlation and control have evolved along with the sequences themselves, and hence are also shaped by the blind trial-and-error processes of evolution. Each enzyme has evolved independently (although in most cases probably by modification from a pre-existing enzyme), and both its catalytic activities and its regulatory characteristics are consequences of its own history rather than features imposed by a grand design. The evolution of each enzyme was guided and constrained by the evolving properties of many others, but such

influences were exerted by functional selection of the whole organism rather than by any general integrating plan. Models, if they are to be potentially useful in the study of metabolic regulation, must follow that same pattern of building up from actual functional relationships rather than attempting to impose a system that is derived from cogitation and based on what are taken to be basic principles.

I think that the most important thing that experimentalists have to say to theorists is to urge them to accept the universe rather than redesign it.

Specific Comments

Kacser & Burns (1973), Heinrich & Rapoport (1974) and their colleagues deserve much credit for emphasizing that enzymes interact in the cell, and that pathways must be considered as functional units. However, they do not emphasize what is possibly the most functionally important type of such interactions, that between the initial enzymes of different pathways that compete for common metabolites. Nor does this model incorporate the fact that most regulatory modifications of enzyme action involve changes in the affinities of enzymes for substrates rather than in catalytic activities or maximal velocities. Similar comments apply to the other major models. Incorporation of those features of real enzymes into models would vastly increase the likelihood that they could prove useful to experimentalists.

More disturbing to experimentalists than models that seem to be irrelevant because of inappropriate assumptions are those that reach specific conclusions about regulation, purportedly on the basis of rigorous analysis, that are self-evidently incorrect. Two recent examples will be offered as illustrations.

Crabtree & Newsholme (1987) maintain that the first enzyme of a metabolic sequence must be saturated with its substrate. That surprising conclusion is based on the argument that the concentration of the initial substrate of the sequence will fall continuously, and that saturation of the first enzyme is a means by which flux through the sequence can be independent of that concentration change. The fact that all of us have survived for decades rather than hours is convincing evidence that concentrations of initial reactants (or any others) do not fall continuously. Metabolism is a quasi-steady-state system. Compounds, such as glycogen and fats, that are cyclically stored and depleted in response to varying rates of supply and demand for energy are insoluble, so that their chemical activities do not change as they are generated or degraded. (The location of phosphorylase in the glycogen granule probably eliminates even the small effect that might be thought to result from a decrease in the surface area of the glycogen particle that is available to the enzyme.) Not only is the premise on which the conclusion is based invalid, the conclusion flies in the face of nearly all of our knowledge of metabolic organization. As well be discussed later, it seems to be well established that the degree of saturation of the first enzyme is the most important determinant of the flux through most metabolic pathways.

A recent short paper (Sauro & Fell, 1987) points out that variation in the amount of an enzyme that exhibits zero-order kinetics would control the rate of the sequence in which it

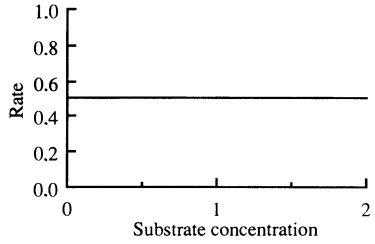


Figure 1. Rate as a function of substrate concentration for a hypothetical enzyme of kinetic order zero.

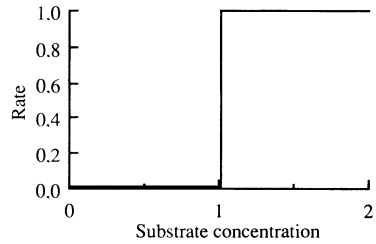


Figure 2. Rate as a function of substrate concentration for a hypothetical enzyme of kinetic order infinity.

occurred, and that variation of the activity of an enzyme that exhibited infinite cooperativity (kinetic order infinitely large) would not have any effect on flux. In their words, the flux control coefficient for the enzyme tends to 1 as the kinetic order approaches zero and tends to zero as the order approaches infinity. They say that they arrived at this conclusion by use of the rigorous theory of Kacser & Burns (1973), implemented in a computer model using a special simulator language, and note that they could alternatively have supplied a formal mathematical proof.

Neither of those approaches was necessary, however. The conclusions are intuitively evident, and can be supported as rigorously by a simple graphical argument as by formal mathematical analysis or computer models. The rate-vs-concentration curves for the (unattainable) boundary conditions of kinetic order zero and of order infinity are shown in Figs. 1 and 2. Zero order (Fig. 1) means that the velocity of the reaction is equal at all finite concentrations of substrate (it must, of course, be zero at zero concentration). An infinitely high order (Fig. 2) means that the concentration of substrate is equal at all rates of reaction. The $S_{0.5}$ value and the saturating concentration are identical in this extreme case. The rate will increase or decrease in such a way as to hold the substrate concentration invariant. It follows that the rate of a reaction catalysed by an enzyme of zero kinetic order will depend only on the catalytic activity of the enzyme. If a sequence with no branches or additional points of input is at steady state, the rate of each reaction is equal to the system flux; thus the amount (or catalytic activity) of a zero-order enzyme would uniquely determine flux. (That would be nearly true also for a real enzyme that was nearly saturated, since the kinetic order of a real enzymic reaction approaches zero as the velocity of the reaction approaches V_{max}.) A large change in the amount of an enzyme of infinite kinetic order (Fig. 2) would not affect the rate of the reaction at all (as long as V_{max} exceeded the flux). Thus such changes could not affect flux through the sequence; the flux control coefficient of the enzyme, as defined by Burns *et al.* (1985), would be zero. For enzymes of high kinetic order, the actual order changes sharply with small changes in rate; if the order were infinite, the change would be abrupt. If the amount of the enzyme were reduced continuously, there would be a point at which the potential rate of supply of substrate exceeded the V_{max} of the enzyme, and at that point the kinetic order would change instantaneously to zero and the flux control coefficient would jump from zero to 1.

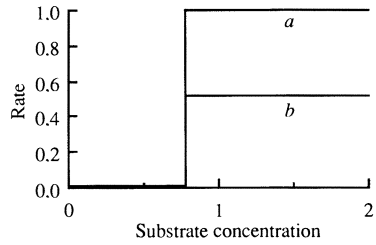


Figure 3. Rate as a function of substrate concentration for a hypothetical enzyme of kinetic order infinity with maximal velocity modulated by a negative modifier metabolite or effector. *a*, rate in absence of modifier; *b*, rate in presence of modifier.

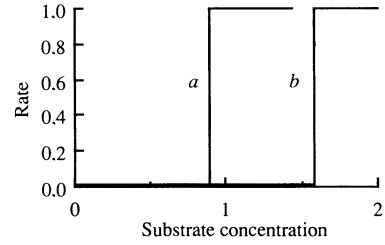


Figure 4. Rate as a function of substrate concentration for a hypothetical enzyme of kinetic order infinity with the affinity of the enzyme for substrate modulated by a negative modifier metabolite or effector. *a*, rate in absence of modifier; *b*, rate in presence of modifier.

From those simple kinetic considerations, Sauro & Fell (1987) point out correctly that enzymes that display positive cooperativity cannot simultaneously have high flux control coefficients as defined. They further assume that this means that such enzymes cannot control fluxes through sequences. They note that this conclusion is in complete contrast to the traditional point of view, according to which allosteric enzymes, which usually possess cooperative kinetics, are the sites of highest flux control.

This example illustrates how the use of erroneous assumptions can lead to egregiously erroneous conclusions. The conclusion that an enzyme with high kinetic order could not have an important effect on flux would be valid if the enzyme were regulated by modulation of its maximal velocity (Fig. 3). A 50% decrease in V_{max}, from *a* to *b*, would have no effect on flux, as long as the new value of V_{max} exceeded the flux allowed by other components of the sequence.

However, as far as I know, no enzyme that catalyses a reaction of high kinetic order has been observed to be regulated by modulation of V_{max}. They all respond to modifiers by changes in the value of $S_{0.5}$, the concentration of substrate at which the reaction velocity is half of that at saturating concentration. (For reasons of logical consistency and dimensional accuracy, the symbol K_m is not appropriate for enzymes with kinetic orders either larger or smaller than 1.) If the $S_{0.5}$ value of the hypothetical infinitely cooperative enzyme is increased by a factor of 2 (from *a* to *b* in Fig. 4), the reaction will stop completely. If the sequence were sealed off from either gains or losses from or to the outside, and if this enzyme catalysed a reaction late in the sequence, as assumed by Sauro & Fell (1987), the concentration of substrate would merely build up to the new value of $S_{0.5}$, after which the enzyme would again have no effect on flux. But no known metabolic sequence is like that. Enzymes with high-order kinetics are always, as far as I know, immediately at branch points (or are one step away from a branchpoint and are linked to the branchpoint by a reaction that is in equilibrium, so that in effect the regulatory enzyme is at the branchpoint even in such cases). If the concentration is at a value between the vertical parts of curves *a* and *b* in Fig. 4, it is obvious that a small change in $S_{0.5}$ can cause the flux to change from zero to V_{max}.

Rather than the step function illustrated in Figs. 3 and 4, real regulatory enzymes exhibit sigmoid curves of rate as a function of substrate concentration, as in Fig. 5. The orders are

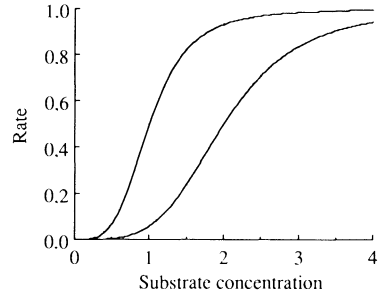


Figure 5. Rate as a function of substrate concentration for an enzyme that binds substrate with a high degree of cooperativity to four catalytic sites, with the affinity of the enzyme for substrate modulated by a negative modifier metabolite or effector. *a*, rate in absence of modifier; *b*, rate in presence of modifier.

typically between 2 and 4; the curves of Fig. 5 are calculated for fourth-order reactions. It is evident that small changes in $S_{0.5}$ can still cause very large changes in reaction rate in such cases. Although the flux control coefficient as defined by Burns *et al.* (1985) is low for such enzymes (since a change in amount of enzyme would have little effect on flux), the flux of the sequence is probably controlled almost entirely by modulation of the $S_{0.5}$ value for the first enzyme. The flux control coefficient of an enzyme, defined in terms of changes in V_{max} or amount of enzyme, is almost entirely unrelated to its regulatory importance — that is, to the extent to which it actually controls flux. Sauro & Fell's erroneous conclusion resulted from their assumption that flux control coefficients as defined in the model are measures of the extent of control. Since very few enzymes are modulated by change in V_{max} or catalytic activity, which is the parameter on which flux control coefficients are based, an enzyme might have a flux control coefficient close to zero but still exert nearly 100% of the control of the flux through the sequence. That indeed is probably the usual situation for nearly all enzymes of regulatory importance. It is noteworthy that high kinetic order, the feature that allows an enzyme to function as a powerful and sensitive determinant of flux when its $S_{0.5}$ is modulated, is the same property that causes an enzyme to have a low value of the flux control coefficient of Burns *et al.* (1985), which is based on the assumption that V_{max} is the modulated parameter.

A kinetic order of zero, or some low finite value, would be useful for stabilizing rates at the expense of wide excursions in concentrations. A high kinetic order is useful for minimizing changes in concentrations while allowing for large fluctuations in flux. Since the need in metabolism, as well as the observable reality of metabolic regulation, is for velocities to be regulatable over wide ranges, as in the transition between rest and heavy exercise, while concentrations are stabilized closely to avoid disruptions of related sequences, it is obvious why regulatory enzymes in general are characterized by high kinetic orders.

Because they are located at the beginnings of sequences (that is, at branchpoints) and because they are regulated by modulation of $S_{0.5}$ rather than V_{max}, high-order enzymes are the main control elements of metabolic sequences. The fact that the erroneous conclusion of Sauro & Fell (1987) was reached with the aid of the model of Kacser & Burns (1973) illustrates the fundamental differences between the assumptions of that model and the characteristics of real metabolic regulatory systems. The assumption that all enzyme modulations are equivalent to changes in amount of enzyme sharply differentiates that model, and also many others, from actual metabolic systems.

Kacser & Burns (1973) and other theorists are correct in pointing out that there is no underlying chemical necessity that one reaction in a sequence should exert primary kinetic control. On the other hand, there is no difficulty in designing a chemical system in which kinetic control will be concentrated at one reaction. That appears to be the situation in the evolutionarily-designed sequences of metabolism. Highly-sensitive partitioning at branch-points determines the rates of initial reactions and thus the fluxes through pathways. If the enzymes that catalyse later steps are present at levels assuring that their V_{max} values will exceed the highest flux that is permitted by the first enzyme, effective kinetic control will reside exclusively at the branchpoint.

Because it is short and clearly written, the paper of Sauro & Fell (1987) illustrates especially well three features that experimentalists would strongly urge theorists to avoid:

1. Unnecessarily complicated treatment of essentially simple concepts.
2. Careless choice of assumptions, which result in conclusions that are not valid.
3. An intellectual value system within which it seems self-evident that a few hours of mathematical doodling by a theorist can invalidate any amount of work and thought by experimentalists.

Being human, experimentalists are likely to be put off by feature 3 — not so much because of the claim that they are wrong as by the implication that their work is so trivial that it would not be worth a few minutes of a theorist's time to consider whether it might perhaps have some validity. Being busy and, in most cases, out of practice mathematically, they are likely to be prevented by feature 1 from ploughing through elaborate models. But of course it is feature 2 that can constitute an insuperable bar to useful interaction between theorists and experimentalists. Models that lead to grossly erroneous conclusions can neither aid experimentalists in planning experiments nor contribute to better understanding of metabolic regulation by anyone.

As a point for discussion, one could suggest that mathematical models have not as yet contributed either to experimental design in metabolic biochemistry or to understanding of metabolic regulation. Some participants may disagree with that statement. Others will consider it to be irrelevant to their interests, which have no room for consideration of actual systems. But for those, of whom I am one, who share the hope of the organizers of this Symposium that a common ground can be found between theorists and experimentalists, the present situation is discouraging. As yet, there has been little apparent interest on either side in building bridges. Improvement will depend on the realization that both experimentalists and theorists have something to offer. Theorists can supply a stronger mathematical orientation and presumably an inclination toward broad interpretations. Experimentalists can supply information on the actual properties of enzymes and on the organization of metabolism, without which models can be only interesting mathematical diversions. It is to be hoped that at some time collaborations between theorists and experimentalists, with intellectual input from both, will lead to experimental tests whose results can feed back and contribute to the evolution of more relevant models that will actually lead to increased understanding of the mechanisms of metabolic regulation.

Design of Models that are Relevant to Metabolic Regulation

I will end by listing some of the conclusions from experimental work on metabolism and on regulatory enzymes that seem to me to be most directly relevant to the design of models for metabolic regulation.

Metabolism is a complex network, and regulation must logically be focussed on branch-points. Most biosynthetic sequences and most regulatory segments of the major catabolic sequences appear to be regulated in large part by modulation of the $S_{0.5}$ value of an enzyme of high kinetic order that catalyses the first step of the sequence or segment. Such steps are, of course, at branchpoints. When the branchpoint is one at which a relatively low-flux branch, such as a biosynthetic sequence, diverges from a mainstream high-flux sequence such as glycolysis or the citrate cycle, the concentration of the branch-point metabolite will probably not be affected significantly by the drain into the biosynthetic branch, so the situation is illustrated approximately by Fig. 5. If the concentration of substrate is in the range between about 1 and 1.5, it can be seen that a 2-fold change in $S_{0.5}$ (illustrated by the curves of Fig 5) can cause the flux through the biosynthetic sequence to vary by a factor much larger than 2. In contrast, a 2-fold change in V_{max} could not alter the flux by more than a factor of 2. Enzymes with high kinetic orders with respect to substrate, and with their affinities for substrate (the reciprocal of $S_{0.5}$) modulated by appropriate metabolic signals, would appear to offer the greatest possible sensitivity of control in response to small signals, and that is the kind of control that has evolved for most sequences. It is evident from our discussion in connection with the paper of Sauro & Fell (1987) that V_{max} control of an enzyme with high kinetic order would be ineffective, so it is not surprising that enzymes with such features have not evolved.

When the metabolic context of a branchpoint is such that the fluxes through the two branches are approximately equal, as when a biosynthetic sequence forks, the situation is more complex. The simplest such case is illustrated in Fig. 6. If the initial properties of the competing enzymes are represented by curves *a* and *b*, the branch that is initiated by reaction a will obtain most of the branchpoint metabolite and its flux will be much larger than that of the other branch. If the $S_{0.5}$ value of enzyme *a* is increased four-fold (curve *a**) by the action of a modifier, the flux through the branch initiated by reaction *b* will be

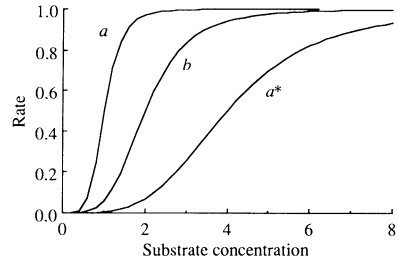


Figure 6. Effect of modulation of $S_{0.5}$ on the competition between two enzymes for a common substrate. The figure shows rate as a function of substrate concentration for two enzymes, *a* and *b*. The $S_{0.5}$ value for *b* is twice that for *a*. In consequence, the rate of the reaction catalysed by enzyme *a* is much greater than that of the reaction catalysed by enzyme *b* at substrate concentrations where neither enzyme is saturated. When $S_{0.5}$ for enzyme *a* is increased four-fold by a modifier metabolite (curve *a**), the rate of the reaction catalysed by enzyme *b* will be much greater than that of the reaction catalysed by enzyme *a*.

much larger than that through the branch initiated by reaction a. The ratio of fluxes through the branches may change by a factor of well over 100.

The concentration of the branchpoint metabolite is likely to change in response to changes in affinities of the competing enzymes. That may either increase or decrease the sensitivity of the partitioning, depending on the relative values of the parameters of the system. In addition, in some cases the affinities of the two enzymes will vary in opposite directions in response to the same metabolic situation; that will obviously greatly increase the sensitivity of the system. If two fourth-order enzymes compete for a common substrate, a 2-fold increase in the $S_{0.5}$ value for one and a 2-fold decrease in the $S_{0.5}$ of the other can also change the ratio of fluxes through the two branches by a factor well over 100. Changes of such magnitudes are common in metabolism; it would be difficult to explain how they could be generated in systems in which V_{max} was the modulated parameter.

In general, the rate of an enzyme-catalysed reaction could be controlled by changes in the amount of the enzyme, by modulation of the catalytic constant, the turnover number, or V_{max} (which are equivalent terms that relate to the rate of catalysis by a filled site), or by a change in the fraction of the catalytic sites that are occupied by substrate. Control by modulation of V_{max} is rare. There are changes in the amounts of enzymes in cells, but such changes appear usually to be related to the need for enough enzyme to meet maximal needs, and not to contribute directly to moment-by-moment regulation of fluxes.

Nearly all metabolic regulation appears to be exerted by control of the degree of saturation of enzymes — the fraction of catalytic sites that bear substrate molecules. The ratio of filled to empty sites is, in the generalized Michaelis-type treatment that is probably a good approximation for the great majority of enzymes, equal to $(S/S_{0.5})^h$, and the fraction of sites filled is $[S^*)^h/(1+(S^*)^h]$, where $S^* = S/S_{0.5}$ and h (the Hill coefficient) is a measure of the number of sites and the degree of cooperativity of binding. In the laboratory, workers usually control the degree of saturation by varying S, the concentration of the substrate. The degree of saturation of regulatory enzymes in the functioning cell appears to be controlled primarily by modulation of $S_{0.5}$. It appears to an experimentalist that models, if they are to contribute to understanding of metabolic regulation, should reflect that fact. Modification of models to incorporate more realistic control mechanisms based on modulation of $S_{0.5}$ values would offer much more potential aid to experimentalists in designing experiments and thinking about the meaning of their results than can the present models.

Discussion[1]

JEAN-PIERRE MAZAT

I should like to begin by answering the last statement in Atkinsons's introduction: for me, the most important thing that theoreticians have to say to experimentalists is to urge them to work out themselves the theory they need. Control theory provides a good example of this, being developed initially by Kacser, a geneticist, to explain results from genetics, and

[1]EDITORS' NOTE. The points raised in the Discussion are considered by Atkinson at the end of Chapter 36.

subsequently exploited by bioenergeticists to resolve conflicting results from different laboratories concerned with oxidative phosphorylation.

The reproach to theoreticians about "redesigning the universe" is an old one, and in many cases I believe it to be unfair, being put forward by some people as a way of avoiding discussion of new theoretical concepts, sometimes difficult ones. There are, of course, many unrealistic theoretical developements, just as there is much irrelevant experimental work. In both cases, however, I think the only reasonable attitude is to ignore them; other attitudes lead to a great waste of time.

I now comment on the three Commandments that Atkinson believes theoreticians must obey when working with experimentalists :

1. I completely agree with point 1. As a teacher, I have always thought that simple concepts can be illustrated by examples from real life, and I have tried to do that with control theory (Mazat, 1988)]. But what is a "simple treatment"? Is matrix algebra simple, for example? A mathematician would certainly say yes, but a biochemist working with mitochondria might well think (wrongly) that the mathematician's matrix is much more complicated than the mitochondrial matrix!

   This emphasizes another point : for theoreticians and biologists to work together they must speak the same language, or at least try to understand what the other says. For this reason I believe there is a need for "cross teaching", i.e. teaching of mathematics to biologists and biology to theoreticians. The Société Française de Biologie Théorique has tried to foster this for the past eight years, with the next school to be held in 1990 during three weeks at Solignac (near Limoges). Several such schools have been held already; they have promoted many collaborations between theoreticians and experimentalists.

2. I disagree with point 2. First, it can be useful to arrive at an invalid conclusion as, properly interpreted, this provides a way of identifying and discarding a bad choice of assumptions. This is a way in which theoretical modelling can be used. Second, the experimentalists' assumptions are not always clear even for themselves, let alone their readers. They are rarely written down, whereas for theoretical work the assumptions have to be explicit, and sometimes this is the first time they are made explicit. At that point a need arises for thorough discussion between theoretician and experimentalist: what are the basic assumptions of the experimental work? what is its history? why was the particular work done? in the way in which it was done? ... This is not an easy discussion, nor always a pleasant one.

3. Of course, I agree with point 3.

I disagree completely with the remark beginning "As a point for discussion...". Figs 5 and 6 of Atkinson's paper were drawn by computer, and this must have been done on the basis of a model to provide the equations; I would guess that the model of Monod *et al.* (1965) was used. Models of this sort, beginning with that of Hill (1910), have been of great help for explaining the experimental results obtained with regulatory enzymes: the existence

of oligomers, conformational changes, cooperativity, etc. They have predicted experimental behaviour that has been tested experimentally.

I would like to give some examples of applications of control theory that have illuminated experimental results in metabolism. One is its application in genetics to explain the vast occurence of recessive mutations in genes coding for the enzymes involved in intermediary metabolism [see the beautiful paper of Kacser & Burns (1981) on the molecular basis of dominance]. This is the first simple explanation of this long standing riddle; they show that "the observation of almost universal recessivity of Mendelian mutants, far from constituting a problem requiring an evolutionary explanation, is seen to be a necessary consequence of the summation theorem".

Another example is provided by the application of control theory to the control of oxidative phosphorylation. Before the work of Groen *et al.* (1982) in Amsterdam there was no agreement among workers about which step (supposedly unique) controlled oxidative phosphorylation in mitochondria. Applying the theory of Kacser & Burns (1973) and of Heinrich & Rapoport (1974) they showed that the control of this system could depend on several steps and *also* on the particular steady state being studied. One can say that this application changed the way in which we now view mitochondrial metabolism.

There are many other applications of metabolic control theory, and there are also, I believe, other theories that have been of great value for interpreting experimental results. I hope that I have shown that one does not have to be as pessimistic as Atkinson in the domain of relationships between theoreticians and experimentalists. These relationships are not easy, but when they succeed they can change the way in which we view the experimental world. This is especially true of metabolic networks, where the great number of variables and parameters involved prevents the use of intuitive reasoning.

DAVID A. FELL and HERBERT M. SAURO

Atkinson's working paper presents an artificial dichotomy between experimentalists and theorists. Darwin (1903) noted "About thirty years ago there was much talk that geologists ought only to observe and not to theorize... How odd it is that anyone should not see that all observation must be for or against some view if it is to be of service!" We doubt that anyone nowadays would argue against Darwin that there is a significant role in science for pure observation uncontaminated by any theory or hypothesis, and in one major school of the philosophy of science (Popper, 1959), the essential characteristic of science is to perform experiments that could potentially falsify a hypothesis.

Atkinson himself uses model and hypothesis to describe the features of "actual systems" that theorists must take into account for their studies to have relevance for experimentalists. For example, he states: "Most enzymes are non-regulatory in the usual sense, and their behaviour is adequately described by the familiar Michaelis equation". Yet the Michaelis-Menten equation itself is a model; it is derived for the simplest reaction scheme sufficient for the purpose and is solved, approximately, for the case of a single-substrate enzyme acting in the absence of product. Since some three quarters of enzymes have two or more substrates,

and all are reacting in the presence of their products when in a metabolic pathway, it is clear that recommending the use of the Michaelis-Menten equation as a model of an enzyme in a pathway involves some subjective judgement about what is "adequate description". An example of an error arising from the incorrect application of the Michaelis-Menten model in interpretation of experimental observations was shown by Boscá & Corredor (1984). They concluded that glycolysis must be regulated below phosphofructokinase because the concentration of fructose 1,6-bisphosphate in certain cells is much higher than the K_m of aldolase towards this substrate. This implication that aldolase is saturated ignores the concentration of the two products of the aldolase reaction and the equilibrium constant, which are such that neither is the enzyme saturated with its substrate, nor are its kinetics rectangular hyperbolic with respect to substrate.

Atkinson also charges theorists variously with using oversimplified models at one extreme and hard reductionism at the other. A model is only useful if it can represent some significant feature of the real system but is simpler to understand and manipulate. Thus it might be useful to construct a model of metabolism using linear kinetics to investigate whether a particular phenomenon can be exhibited by such a model, or whether the intrinsic non-linearity of enzymic catalysis plays an indispensable role. Any model must represent a compromise between extreme abstraction, giving a poor approximation to the real system, and over-elaboration, giving greater generality and realism in its application at the expense of comprehensibility. This, however, is a problem for us all, as "experimentalists" use models in their explanations just as "theorists" do, as we attempted to illustrate in the previous paragraph; it is just less obvious in the former case as the models are often verbal rather than mathematical, and their development is less formal. Our common concern is to decide which models are useful, and which are unhelpful or misleading.

One of Atkinson's specific criticisms is directed at our abstract (Sauro & Fell, 1987): its conclusions could have been reached by an alternative route. We cannot contest this, because it is invariably the case that different formalisms can be applied to a particular problem and yet yield compatible results. However, different routes to the same explanation may differ in their rigour, or their comprehensibility for a particular audience. We would suggest that whilst his approach has some heuristic value, it lacks the rigour that he claims. A significant difficulty with his analysis is that it attempts to define the range of behaviour of metabolic systems at steady state by describing the extremes. However, the infinitely cooperative system (Fig. 2) cannot give rise to a steady state in a pathway for a flux value less than the V_{max}, and therefore its validity as a model of steady-state behaviour is uncertain.

However, our major point is that his claim that our conclusions were in error and that the theory of Kacser & Burns (1973) has limited relevance to real systems is based on a complete misrepresentation of the basis and applicability of metabolic control analysis. His argument is based on repeated assertions and implications that the uses of flux control coefficients are limited to their formal definition, i.e. that as they are defined as the response of a metabolic flux to variations in an enzyme activity (in other words, to its V_{max}), they can only be used to determine the response of flux to factors that affect the V_{max}. Even if this were true, it would still allow metabolic control analysis to apply to regulation by induction

and repression of gene expression and by covalent modification of enzyme activity, which are both ignored by Atkinson when he attributes most regulation to modulation of $S_{0.5}$ values of branch-point enzymes. However, the flux control coefficient is a component of the "response coefficient" described by Kacser & Burns (1973; see also Kacser & Porteous, 1987): the response of a system flux J to an effector Q acting on enzyme i is the flux control coefficient of enzyme i on J times the elasticity coefficient for the rate of enzyme i with respect to the metabolite Q, i.e.

$$R_Q^J = C_i^J \varepsilon_Q^i$$

This is true whatever the functional form of the effect of Q on enzyme i; in other words, it is true when Q acts as a K-system effector of "i", and therefore applies to changes in $S_{0.5}$ (Fig. 4). [The use of the response coefficient for changes in "K" parameters has been illustrated by Westerhoff & Kell (1987.)] The equation shows that however strong the effect of Q on "i", it can have no effect on the flux through the system if C_i^J is zero, since R_Q^J will necessarily be zero. In this sense, therefore, Atkinson's statement that "an enzyme might have a flux control coefficient close to zero but still exert 100% of the control of the flux through the sequence" cannot be true for realistic values of ε_Q^i. If he can give a quantitative definition by which "100% of the control" can be objectively measured, we are prepared to determine the control coefficients for any model system that he can propose that is claimed to exhibit this property along with a very low flux control coefficient, and to report the results to the meeting.

In summary, our position is that metabolic control analysis already contains the features that Atkinson seeks: its applicability is not affected by the functional form of enzyme kinetics (i.e. it works whether the kinetics are linear, Michaelian, sigmoidal or of some other form, because of the general nature of the definition of the elasticity coefficient); it can be used to describe the response of systems containing K-system feedback loops, and it is applicable to branched and cyclic systems. We hope to illustrate this in our own contributions to the symposium (see Chapter 9).

MARK SALTER and RICHARD G. KNOWLES

Although we found the Atkinson's working paper stimulating, we believe that he has failed to appreciate two very important points concerning regulation and control theory; and because of this we believe the major part of the arguments are flawed.

Regulability versus control coefficient. It is an unfortunate fact that the nomenclature in the literature generally is unclear with regard to the meanings of regulation, regulability and control, and this has led to considerable confusion. Perhaps it would be less confusing if the term "control" was used to denote control coefficients, "regulability" to denote the degree to which enzyme activity can be changed by any effector; and the "regulatory importance" of an enzyme would be a property derived from these two factors together, and would therefore define an enzyme to change pathway flux (or a metabolite in that pathway). The regulatory

importance of an enzyme is therefore a combination of *both* its control coefficient and its regulability, as has been emphasized previously by Crabtree & Newsholme (1985), by ourselves (Salter *et al.*, 1986) and by others. The importance of this point in relation to Atkinson's paper will be discussed below.

V_{max} versus "activity under pathway conditions" (v). Although one of the originating papers of control theory emphasized control coefficients as related to changes in V_{max} of the enzyme under study, other work then and since has used the more general expression relating changes in flux (or metabolite concentrations) to changes in v, where v is the net activity of the isolated enzyme under the original pathway conditions (i.e. the substrates, products and effectors at their concentrations found experimentally). Expressing control coefficients as a function of v rather than V_{max} allows for the fact that pathway fluxes (or metabolites) can be modulated by changes in *either* K_m or V_{max} of an enzyme.

Implications for Atkinson's paper. Atkinson concludes that the control theory should take into account changes in $S_{0.5}$. In fact, with the use of v the present theory already takes changes of $S_{0.5}$ into account. The example given in Fig. 6 shows an enzyme (a/a^*) of low control coefficient but with very high regulability (although $S_{0.5}$ only changes 2-fold the v at $S = 1.25$ changes approximately 100-fold). Because of the consequent regulatory importance of this enzyme, the paper concludes that the flux control coefficient of this enzyme "is almost entirely unrelated to its regulatory importance", implying that the flux control coefficient is not a useful piece of information. However, as discussed above, determination of the regulatory importance of an enzyme requires knowledge of both the regulability and the control coefficient and also the way in which the control coefficient changes over the range of regulation. For example, if an enzyme had a flux control coefficient of 0.0001 then a 100-fold increase in v would not produce a significant change in pathway flux; a 100-fold decrease in v might have a significant effect, but only if the control coefficient of the regulated enzyme increased by an amount that allowed the change in v to significantly decrease flux (or a pathway metabolite).

Other points. 1. The theoretical infinite kinetic models used in the paper are so divorced from real systems as to be unhelpful: they imply that a certain concentration of S the enzyme will be infinitely regulable. It is illogical to extrapolate from a model of infinite kinetic order to a system of finite kinetic order.

2. Atkinson states that the flux control coefficient of an enzyme of high kinetic order situated at a branch point would have a low flux control coefficient. This is not necessarily the case, as one may see by examining a system in which enzyme *a* carries 100% of the flux into a branch out of which enzyme *b* carries 99% of the flux and enzyme *c* carries 1%. If *b* was far from saturation then *c* would have a high flux control coefficient for its own flux. Atkinson also states that the flux through this enzyme would probably only be "controlled" (in our terms, regulated) by modulation of $S_{0.5}$ of the same enzyme.

Again, this is not necessarily true. If *c* is followed in linear sequence by enzyme *d*, and changes in the v of *d* (for example by altering either its K_m or its V_{max}) changed the concentration of the metabolic intermediate between *c* and *d* in a range where this intermediate had a significant inhibitory effect on *c*, then *d* could have a large flux control

coefficient and could therefore (given significant regulability) regulate the pathway *c-d*.

It would seem from our comments above that the way to advance the quantification of "regulatory importance" would be to formulate a theory combining control coefficients and regulability of enzymes. At present this would appear to be difficult to attain, given that it would first be necessary to formulate a theory describing how control coefficients change with changes in *v*.

ATHEL CORNISH-BOWDEN

I think the suggestion of Drs. Salter and Knowles about the distinctions to be made between *regulation*, *regulability* and *control* is a very valuable one, and believe that if it were widely adopted it might help to resolve some of the confusion that currently exists in control theory. Studying control would then involve measuring and reporting control coefficients (logically enough), whereas studying regulability would involve elasticity coefficients. In time, one might suggest that *regulability coefficient* would prove a more evocative (and therefore more comprehensible) term than *elasticity coefficient*, but such a short time after the latter became common it is probably premature to propose a replacement. In general, the suggestions made by Drs. Salter and Knowles have the great merits that they do not conflict (as far as I can see) either with existing usage or with the meanings that one might attach to the terms if one came upon them out of context with no precon-ceptions about what they might mean.

Finally, I agree very much with their view that we need to develop a theory, and indeed a measure, of *regulatory importance* that combines in some way the ideas of control coefficients and regulability. This might be very helpful in resolving the paradox whereby the effect of feedback inhibition of an enzyme is to *decrease* its flux control coefficient for the flux through its own pathway. One might hope that a measure of regulatory importance would be increased by the occurrence of allosteric inhibition. I suspect that confusion between control coefficients and regulatory importance is the source of some of the resistance many biochemists still have towards adopting the ideas of metabolic control theory.

JAN-HENDRIK S. HOFMEYR

I read Atkinson's pre-conference working paper with great interest since I, like many other biochemists, have always found his papers and especially his book *Cellular Energy Metabolism and its Regulation* (Atkinson, 1977) particularly lucid and thought-provoking. I have now received a copy of his recent book *Dynamic Models in Biochemistry* (Atkinson *et al.*, 1988) and am glad that I waited for that before writing my answer. Besides being an extremely useful and creative book it has also given me a better idea of how Atkinson views the control of metabolism. Although I agree wholeheartedly with many of his statements, I do have reservations about others. These comments address some these not already dealt with by other contributors to this Prologue.

It seems as if there still exist two schools of thought about metabolic control, let us call

them the "old" and the "new" school for want of better words and without implying that either is all correct or all wrong. Let us say that the old school is embodied in what one would read about metabolic regulation in a general biochemistry textbook and the new, for instance, in the writings of Kacser & Burns (1973), Heinrich & Rapoport (1974), and Savageau (1976). Crabtree & Newsholme (1985), also influential writers on metabolic regulation, would stand (perhaps uncomfortably) with one foot in each camp. In a sense Atkinson seems to embrace the old school, although his is a particularly well informed view of metabolic regulation. I, on the other hand, subscribe to the new school, but would not for a moment disregard the basic common sense in a lot of Atkinson's statements and intuitions. Let me immediately say that the two schools are by no means incompatible and we should strive to combine their strongest features into a coherent whole. (Note that I do not distinguish between theorists and experimentalists, a distinction that, in my view, does not contribute much to the debate. To my knowledge most of the workers seriously interested in metabolic control use both theory and experiment.)

Much of the misunderstanding that exists between the two schools seems to me to be of a semantic nature, and, sadly, because the old school seems not to have really made the effort to understand what the new school is saying. I shall try to clarify a few of the semantic matters and general misunderstandings in the hope that we shall be able to discuss really important things at the workshop and not waste time quibbling about the meanings of words. For those who do not want to wade through my arguments, the points I wish to make can be summed up as follows:

1. It is misleading to talk of the "model" of Kacser & Burns (1973). They did not propose a new model of metabolic processes, but rather developed a quantitative language to talk about the properties of the basic kinetic model of metabolism that has been in use for decades, the same model that Atkinson uses.

2. Whereas the old school tends to use the word "control" in a general way (in much the same way as we use it in everyday language), it has acquired a specific meaning in the framework of the new school. This has evidently caused much confusion.

3. Although in most publications the definition of control coefficients is based on the measured response of a flux or concentration to change in *enzyme concentration*, a more general definition in terms of a change in *local rate* is less confusing in that it does not lead to the idea that control analysis only considers changes in V_{max} and not changes in, for example, $S_{0.5}$.

Metabolic models

In the working paper Atkinson often uses the word "model", but his use of the word is rather loose and I suspect that this may cause confusion. He does, however, raise important questions such as: What aspects of real metabolic systems should a model include to make it

relevant? Do we lose, in the quest for generality, our concern with real properties of real systems? He talks about the "model" used by Kacser & Burns (1973) and how this purported model does not include control of enzyme action through modification of affinity for substrate, but supposes that all enzyme modulations are equivalent to changes in the amount of enzyme. I do not intend philosophizing about models and modelling, but clearly all of this needs comment.

First, although Atkinson claims pie-in-the-sky status for models built from so-called first principles, both his and our own models must clearly be based on something. Let us find common ground between the two schools. Surely both basically view metabolic systems as networks of enzyme-catalysed reactions and transport steps linked to each other by mass action (the product of one step being the substrate of the next). There are, of course, other mechanisms of metabolite-enzyme and enzyme-enzyme interaction, a common one being the regulatory loop (a metabolite that is neither a substrate, product nor a cofactor interacts with an enzyme and changes its activity). For the sake of simplicity both schools usually assume "free pool" status for the intermediary metabolites, i.e. there are no diffusion gradients within the system (the chemical engineer's well-stirred reactor) and the metabolites are not channelled. This view leads naturally to what I would call the *basic kinetic model* of metabolism: the rate at which the concentration S_i of an intermediary metabolite changes is the sum of the rates v_j that synthesize that metabolite minus the sum of the rates that consume it. For a system with n enzymes the basic kinetic model can be represented mathematically as a system of differential equations, one for each of m metabolites:

$$\frac{dS_i}{dt} = \sum_{j=1}^{n} c_{ij} v_j \quad \text{for } i = 1 \cdots m$$

The coefficients c_{ij} refer to the stoichiometry of metabolite participation in a reaction (negative for substrates, positive for products and zero for non-participants) and therefore define the mass-action network structure of the system. Both schools usually consider the *steady state* as a starting point: each differential equation equals zero while each individual v_j has a finite value and therefore the net rate of consumption of each metabolite equals the net rate of production; the metabolite concentration is time-invariant; the terminal metabolites are regarded as clamped by the environment of the system. In its simplest form the model assumes that the steady state is both *dynamically stable* (after a perturbation in any of the variable metabolites the system relaxes to the same steady state) and *structurally stable* (after a perturbation in one of the parameters the system relaxes to a closely neighbouring steady state).

Although many readers may find the above obvious, it is important for my argument. I shall assume that both schools use the basic kinetic model as a point of departure. When we discuss the relevance of various metabolic models we usually argue about modification of the basic model, most often assumptions made about the rate equations, i.e. the equations that replace v_j in the model. While it is true that a model that uses only first-order rate equations can give only limited information about the behaviour of a real metabolic system, it is a useful first approximation, if only because it is mathematically more tractable. Atkin-

son seems to think so himself, as in his new book (Atkinson *et al.*, 1988) his first digital experiment on the kinetics of sequential reactions is done on exactly such a model and, in the process, a few extremely important principles about the behaviour of the concentrations and fluxes in such systems are discovered. Of course, this is only a first step and the next step must include saturation properties, variable kinetic orders, etc. In general, both the new and old school accept and apply the above, and both would criticize models that try to explain metabolic behaviour solely on the basis of limiting assumptions. For example, I think that most of us would disagree with Newsholme's insistence that the first enzyme of a pathway has to be irreversible and saturated as a precondition for the existence of a steady state (Crabtree & Newsholme, 1985). At the same time we would agree on the irrelevance of some of the metabolic models that have been analysed in the literature, where no account is taken of the real properties of enzymes.

I am not sure whether Atkinson regards the basic kinetic model as too general, but without any further assumptions about the reversibility, distance from equilibrium, degree of saturation or form of the rate equation, that is the model on which the theory of the new school is based — the theory is a set of mathematical consequences of a specific type of differential analysis of the basic kinetic model. Control analysis and biochemical systems theory provide mathematical languages for making quantitative statements about the behaviour of a metabolic system. Far from compromising their usefulness for the sake of generality, these frameworks provide for the natural inclusion of real enzyme properties; they do not make prior assumptions that limit the properties of the model (an intrinsic problem of some old-school models, where, for instance, first steps are often *defined* as irreversible and rate-limiting and therefore assumes one of the very things that should be under investigation). Any structure is allowed (linear chains, branched chains, loops and moiety-conserved cycles) and, in principle, any molecular interaction can occur. To say that "nor does this model, I believe, incorporate the fact that most regulatory modifications of enzyme action involve changes in the affinity of enzymes for substrates rather than in catalytic activities" is to underrate the theory (more about this later).

So, while I would agree that the aim for generality may be taken too far, a basic metabolic model and the language for describing its behaviour must be general enough to allow for the inclusion of all of the real properties of the functional units of metabolism. I believe that the frameworks of the new school fulfil this criterion and, at the same time, are not so general that no meaningful statements can be made about the behaviour of specific metabolic models. Nonetheless, I agree with Atkinson that when we choose to build and study a specific model on the basis of the basic model we should try to incorporate our present knowledge about real enzymes, real metabolic structures, real regulatory mechanisms, which enzymes are sensitive to feedback, where they usually occur, etc.

To my mind the actual debate about metabolic models is the one that has being going on in the new school, that is, the discussion of how valid the basic kinetic model really is. More and more instances are found of supramolecular associations between enzymes and channelling of intermediate metabolites, so that the assumption that these metabolites exhibit free-pool behaviour may be too limiting. Does this invalidate the frameworks of control analysis and biochemical systems theory? If not, how should it cater for these phenomena?

Are all metabolites channelled? Surely, if the energy charge, $NAD^+/NADH$ or $NADP^+/NADPH$ ratios play an important metabolic role, many enzymes in different pathways must be able to sense their concentrations. Should not at least branch-point metabolites and intermediate metabolites that have feed-back or feed-forward effects on distant enzymes exist as free pools? I hope that these are some of the matters that will be discussed at the workshop.

Momentary Kinetic Orders

In his arguments about metabolic behaviour Atkinson often uses the idea of variable kinetic order. He states, quite correctly, that enzymes are saturable and that their effective kinetic orders change with the degree of saturation. He continues by arguing for a distinction between, on the one hand, "non-regulatory" enzymes that would usually obey Michaelis-Menten kinetics and have a momentary kinetic order described by $1 - v/V_{max}$ (varying between 1 and 0) and, other the other hand, "regulatory" enzymes, that "typically have higher, but still variable, kinetic orders". Is this so? If we agree that momentary kinetic order is defined by $d\ln v/d\ln S$ then only the irreversible Michaelis-Menten rate equation would lead to $1 - v/V_{max}$ as a description of momentary kinetic order. If, however, metabolic reactions are regarded as reversible in principle [and, in an argument for the acceptance of K_m as a measure of affinity, Atkinson (1977) makes a strong case for this in his book] then the momentary kinetic orders of an enzyme with a reversible Michaelis-Menten rate equation are $[1/(1 - \Gamma/K_{eq})](1 - v/V_f)$ for substrate and $[-(\Gamma/K_{eq})/(1 - \Gamma/K_{eq})](1 - v/V_r)$ for product, where Γ is the mass action ratio, v the net reaction rate and V_f and V_r the forward and reverse limiting reaction velocities. These kinetic orders approach ∞ and $-\infty$ respectively as the reaction approaches equilibrium. Would this make them "regulatory" enzymes? Is this classification useful? I leave the argument open.

Regulation and Control

What do we mean when we say that a biochemical system is "controlled" or "regulated"? In the old school this seems to imply the existence of a "regulatory enzyme" or an enzyme with "regulatory importance", e.g. an allosteric enzyme or an enzyme subject to a specific regulatory mechanism of some sort. This I take to be the "usual sense" when I read that "most enzymes are non-regulatory in the usual sense". This statement that only regulatory enzymes typically have variable kinetic orders greater than one I have already discussed. Nowhere could I find a description of how "regulatory importance" could be subjected to quantitative scrutiny which would allow for variation in regulatory power as the metabolic state changes. It seems that, in the old school, an enzyme can only be regarded as potentially regulatory if a *mechanism* exists whereby a metabolite other than the substrates and products can influence the rate of that enzyme. By definition, an enzyme is therefore either regulatory or not. Atkinson (1977) himself pointed that this view is too limited: some

enzymes that show a U-type response have evolved without the development of a separate modifier site.

I have found that the old school view still prevails: On various occasions I have asked either colleagues or students whether the flux in a linear chain of enzymes without a feedback loop is controlled or not. The answer invariably was negative. However, consider the following: A biochemist is confronted by a new organism that takes up a metabolite and transforms it in some way into an excreted metabolite; the rate of transformation (flux) is measured as the rate of production of end-product. When the end-product is already present in the medium the flux is decreased. We would say that the level of end-product "controls" the flux, and would suspect some inhibitory mechanism, perhaps allosteric feedback in which the binding of end-product lowers the affinity of the first enzyme for its substrate. However, a chain of "non-regulatory" enzymes (without a feedback loop) could produce exactly the same qualitative picture. All that is necessary is that each enzyme is sensitive towards the concentration of its own product. If the concentration of final product rises, all the intermediate concentrations rise and the flux will decrease, albeit to a lesser degree than for allosteric feedback. In a similar system where the affinities of the enzyme towards their products are higher, the flux will be more sensitive towards an increase in final product, and will therefore decrease more for a given change in the final metabolite (in all the above I consider small changes). Now, if an experimentalist studies the last mentioned two (non-allosteric) cases, without having any knowledge of whether there is a feedback loop or not, would he not conclude that in the second system the flux is "controlled" more effectively? Of course the existence of a feedback loop increases the potential for control of both flux and concentrations tremendously, but should the concept of control regulation be linked solely to specific molecular mechanisms ?

The new school, on the other hand, uses these words in a much broader but rigorously defined way. All enzymes and various other system parameters can potentially contribute towards control, whether it be control of a flux or of a metabolite concentration. Here the distinction between a parameter and a variable of the system becomes important because control is now defined and quantified as the percentage change in a variable (flux or concentration) resulting from a 1% change in a parameter (such as concentration of end-product, enzyme concentration, affinity towards substrate, product or effector, etc). A change in any parameter can potentially affect, for instance, a flux. So, although both the old and the new school would have to do the same experiments to identify the specific mechanisms in which metabolites and enzymes communicate and interact with each other in, for instance, the above hypothetical system, the new school can at least make quantitative statements about the effectiveness of control by end-product without falling about trying to decide whether the word "control" is applicable or not.

Note what, by adopting the broader use of the terms, we have lost nothing but the special category of "regulatory enzymes". But in the process we do not relegate these enzymes to some lesser category, since we still have a quantitative way in defining their undoubted importance in the behaviour of a pathway. In the broader definition a concept such as allosteric feedback is essentially only a description of an extra mechanism for molecular communication; important, yes, but not a prerequisite for the existence of control.

The broader definition, however, now admits and allows one to quantifiy the regulatory importance of other enzymes; more recent experiments show clearly that they can play such a role. The same applies to many transport mechanisms, which can fulfil an important regulatory function.

Having written this, I have a sneaking suspicion that the old school will still not be satisfied. I sympathize with that, and, risking the wrath of my colleagues, I wonder whether most of the confusion about "to control or not to control" could have been forestalled by not opting for the "control" in "control coefficient". You may ask what's in a name, but I find it a pity that we could not retain the terms "sensitivity coefficient" (although, having opened this old can of worms I dread banishment to the North Pole). It makes more sense to me to say a flux is sensitive to a parameter instead of saying that it is controlled by the parameter — "controlled" easily takes on an all-or-none guise. Control analysis is, after all, the analysis of the behavioural response of a metabolic state to perturbation in a parameter, taking into account all the interactions in the system, "regulatory" or not. I am aware of the history of the current nomenclature, but still feel that I have inherited a rather cumbersome and potentially confusing terminology.

The Usefulness of the Concepts and Constructs of Control Analysis

While Atkinson acknowledges some contributions of the new school to our present understanding of the behaviour and control of metabolism, his main criticism seems to be based on his claim that the theory does not incorporate many of the observed properties of real systems; most important, that it does not allow for enzymes to be controlled by changes in the $S_{0.5}$ values for their substrates, but only by changes in limiting velocity (or enzyme concentration) since this is the parameter on which control coefficients are based. I agree with neither claim nor criticism and would like to show that, by not making any assumptions besides those implicit in the basic kinetic model, control analysis allows for the inclusion of all the realistic enzyme properties mentioned. The only real limitation lies in the type of mathematical analysis that is employed, namely a differential analysis that accurately describes only the effects of small changes in parameters on the steady-state fluxes and concentrations. However, this limitation is forced on us by the inherently non-linear nature of enzyme behaviour.

If Atkinson's claim were true, it would be important since "very few enzymes are modulated by changes in V_{max} or catalytic activity"; in contrast, enzymes are controlled rather by changes in $S_{0.5}$. This may be so, but the statement that "the flux-control coefficient of an enzyme is almost entirely unrelated to its regulatory importance — that is to the extent to which it actually controls flux" indicates the extent of this misunderstanding. Since its introduction, the concept of control coefficient has often been misunderstood, both with regard to what it measures and what it can be used for. The problem, one that has even plagued "believers", is that in most publications by the new school control coefficients quantify a systemic response to a modulation in *enzyme concentration*. This is quite valid while the kinetic order with respect to enzyme concentration is one (which it usually is), but

this definition is obviously prone to misinterpretation and could have led to the type of claim by Atkinson.

In general, a flux-control coefficient simply measures the percentage change in a metabolic variable such as flux or concentration when a 1% change is made in the *local rate* of any reaction *i;* formally it would be defined as $d\ln J/d\ln v_i$ (note that I specifically do not consider a 1% change in enzyme concentration), and such a generalized control coefficient was defined some years ago (Kacser & Burns, 1973; Heinrich *et al.*, 1977). This is a practical definition in the sense that it tells the investigator that he must in some way modulate the local rate of a reaction and then measure the response; the definition does not prescribe how the reaction rate should be modulated in real systems. Now think of a computer-modelling situation: what is the simplest way a modeller can modulate a local reaction rate? One can multiply any rate equation by an arbitrary constant, say α, which if it is assigned a value of unity does not change the value of the rate v. A generalized control coefficient would then equal the percentage change in a flux or concentration in response to a 1% change in α. The introduction of the α parameter is useful for explaining the meaning of a generalized control coefficient but it does not, of course, exist and cannot be manipulated experimentally. Generally, however, enzyme concentration shares with α the property of being a multiplier in the rate equation and it is for this reason that it has crept into the definition of control coefficient. I shall, however, stick to my general definition.

The first important point is that one can consider any mechanism for changing a local enzyme rate (be it by a modulation of enzyme concentration, k_{cat}, $S_{0.5}$ or any other enzyme parameter) and define a control coefficient that quantifies the effect. Let us consider an enzyme *i*, the $S_{0.5}$ of which is sensitive to an external effector, X, that is under our control. We can modulate the concentration of X, measure the change in flux and express the response as follows (this type of coefficient that quantifies a response towards an external clamped concentration is often called a *response coefficient* R_X^J to distinguish it from enzyme control coefficients):

$$C_X^J = \frac{d\ln J}{d\ln X}$$

The so-called "combined response property" of control analysis then gives the relationship between this flux-response coefficient and the generalized flux-control coefficient of step *i* is as follows:

$$R_X^J = C_i^J \varepsilon_X^i$$

where ε_X^i is the elasticity coefficient of local rate *i* with respect to a modulation in *X* under the prevailing steady-state conditions. We could even break the elasticity coefficient down into the product of two terms:

$$\varepsilon_X^i = \frac{\partial \ln v}{\partial \ln S_{0.5}} \times \frac{\partial \ln S_{0.5}}{\partial \ln X}$$

the first of which shows how sensitive the rate is to a change in $S_{0.5}$ and the second how

sensitive $S_{0.5}$ is to a change in X. The combined response equation clearly shows that the final effect of a modulation in X depends both on the value of the generalized control coefficient (which describes the degree to which a specific step in the reaction sequence limits the flux) and the elasticity of that step itself towards a change in X. Furthermore, since R_X^J and ε_X^i are measurable, the generalized control coefficient C_i^J can be calculated. The summation or connectivity equations of control analysis are generally valid if generalized control coefficients are used, and the generalized control coefficients in any such equation can be replaced by the quotient of a pair of linked systemic and local coefficients such as R_X^J/ε_X^i.

In the same way one could write for a modulation in enzyme concentration E_i:

$$C_{E_i}^J = C_i^J \varepsilon_{E_i}^i$$

The value of $\varepsilon_{E_i}^i$ is usually equal to unity (here the generalized control coefficient is equal to the coefficient based on enzyme concentration). It is obvious that the relative flux-responses to changes in X and E_i will depend on the values of the elasticities; if $\varepsilon_X^i > \varepsilon_{E_i}^i$ the flux will be more responsive to changes in X (this will be the case if the enzyme is highly sensitive to changes in $S_{0.5}$, which in turn is sensitive to changes in X). Far from being unimportant, the control coefficient $C^J{}_i$ is central to the whole matter; if it is very small then a modulation in neither enzyme concentration nor $S_{0.5}$ (*via* a change in X) will have much effect on the flux. Therefore, in reasoning about the metabolic effects of a modulation in any enzyme parameter (including $S_{0.5}$), both the immediate local effect of the modulation (elasticity coefficient) and the systemic effects caused by the modulation in local rate (generalized control coefficient) must be taken into account.

Atkinson's Discussion of the Paper by Sauro & Fell (1987)

I would like to add some of my own comments, both on the original paper and on Atkinson's criticism. My main problem with the paper is that it uses deductions based on a specific (and in a certain sense, an unrealistic) metabolic model to draw general conclusions about metabolic control. However, and here I disagree with Atkinson, the fact that the framework of Kacser & Burns (1973) was used to reach these conclusions does not invalidate the framework itself or make it less useful (the fact that we can make dubious statements in English does not lead us to doubt the usefulness of the language).

The problem with the metabolic model used by Sauro & Fell (1987) is that the last enzyme in their pathway was chosen as the one to bind its substrate cooperatively. As both they and Atkinson show, the presence of a downstream enzyme that displays positive cooperativity will transfer flux-control (in the sense of Kacser and Burns) upstream, away from that enzyme. Even if the $S_{0.5}$ is changed, the flux will be unaffected since the substrate concentration will change so as to cancel the effect on the flux. However, if Sauro & Fell (1987) had chosen the first enzyme as cooperative (the natural choice if one takes the pattern usually found in nature into account) and used the following rate equation:

$$v = \frac{V_{max} S^h}{S_{0.5}^h + S^h}$$

the flux-control coefficient would have been 1 (the enzyme is insensitive to changes in its own product concentration and therefore isolated from the rest of enzymes in the chain; changes in any of the downstream rates cannot affect the rate through the first reaction). Therefore their conclusion that positively cooperative enzymes cannot simultaneously have a high flux-control coefficient as defined by Burns *et al.* (1985) depends on their specific model and is not generally true.

Atkinson, however, accepts their conclusion when he argues that "although the flux control coefficient as defined by Burns *et al.* (1985), is low for such enzymes [here the first enzyme in the pathway] (since a change in amount of enzyme would have little effect on the flux), the flux of the sequence is probably controlled almost entirely by modulation of the $S_{0.5}$ for the first enzyme". Now, the first enzyme in this specific pathway can have a flux-control coefficient less than one only when it is sensitive to changes in the concentration of its own product i.e. when it is reversible or product-inhibited. But, Atkinson's whole argument is based on the properties of an irreversible enzyme (which, as I have shown, will have a flux-control coefficient equal to one). The more sensitive the first enzyme in this system is towards the concentration of its own product, the smaller its flux-control coefficient and the smaller the effect on the flux of a modulation in $S_{0.5}$. So, it remains true that allosteric enzymes that are modulated by changes in $S_{0.5}$ are not necessarily efficient flux-controllers; they must still have a high enough flux-control coefficient for the mechanism to be effective. This again illustrates the importance of the combined response property in our reasoning about metabolic behaviour.

Despite my disagreement with some of his ideas, I admire the forthright way in which Atkinson has set them out; I was forced to consider again many things that I take for granted in my thinking about metabolic behaviour and control. In the spirit of his own recommendations above I would like to put forward three features the new school would like the old school to consider:

1. The behaviour of metabolic systems is often more complicated than one thinks; while striving for simplicity is important, one should be beware of "essentially simple concepts" that could lead you up the garden path.

2. Assumptions should always be carefully considered and stated explicitly (Atkinson's and Mazat's rule).

3. Spending a few hours to really grasp (and doodle with) a fruitful mathematical framework could, besides enhancing one's understanding of the behaviour of metabolic systems, point out the gaps in and even invalidate a part of a century's work and thought by experimentalists.

REFERENCES

Atkinson, D. E. (1977) *Cellular Energy Metabolism and its Regulation,* Academic Press, New York

Atkinson, D. E., Clarke, S. G., Rees, D. C. & Barkley, D. S. (1987) *Dynamic Models in Biochemistry,* Benjamin/Cummings, Menlo Park, California

Boscá, L. & Corredor, C. (1984) *Trends Biochem. Sci. 9,* 372-373

Burns, J. A., Cornish-Bowden, A., Groen, A. K., Heinrich, R., Kacser, H., Porteous, J. W., Rapoport, S. M., Rapoport, T. A., Stucki, J. W., Wanders, R. J. A. & Westerhoff, H. V. (1985) *Trends Biochem. Sci. 10,* 16

Crabtree, B. & Newsholme, E. A. (1985) *Curr. Topics Cell. Reg. 25,* 21-76

Crabtree, B. & Newsholme, E. A. (1987) *Trends Biochem. Sci. 12,* 4-12

Darwin, C. (1861) in *More Letters of Charles Darwin* (Darwin, F., ed., 1903) *1,* September 18, 1861, Murray, London

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. & Tager, J. M. (1982) *J. Biol. Chem. 257,* 2754-2757

Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95

Heinrich, R., Rapoport, S. M. & Rapoport, T. A. (1977) *Prog. Biophys. Molec. Biol. 32,* 1-82

Hill, A. V. (1910) *J. Physiol. (Lond.) 40, iv-vii*

Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104

Kacser, H. & Burns, J. A. (1981) *Genetics 7,* 630-666

Kacser, H. & Porteous, J. W. (1987)*Trends Biochem. Sci. 12,* 5-14

Mazat, J.-P. & Jean-Bart, E. (1988) *Biochemical Education 16,* 28-30

Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol. 12,* 88-118

Popper, K. (1959) *The Logic of Scientific Discovery*, Hutchinson, London (translated from *Logik der Forschung,* Vienna, 1934)

Salter, M., Knowles, R. G. & Pogson, C. I. (1986) *Biochem. J. 234,* 635-647

Sauro, H. M. & Fell, D. A. (1987) *Biochem. Soc. Trans. 15,* 234-235

Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts

Westerhoff, H. V. & Kell, D. B.*Biotechnol. Bioeng. 30,* 101-107, 1987

Part I

GENERAL ASPECTS OF CONTROL THEORY

Chapter 1

The Nature and Role of Theory in Metabolic Control

ATHEL CORNISH-BOWDEN

CURRENT discussion of metabolic control is dominated by the theory developed from the landmark papers of Kacser & Burns (1973), and Heinrich & Rapoport (1974), which built on earlier work of Higgins (1963, 1965). Although the main ideas in this theory have become much more widely accepted by biochemists as a whole in the past few years, acceptance is far from universal, and criticisms have come from various directions. Some of these are set out and discussed in other chapters of this book, but in order to form a judgement, whether about the usefulness of metabolic control theory for analysing real metabolic systems or about its status as a special case of biochemical systems theory (Savageau, 1969*ab*, 1970, 1976), as it is categorized by Savageau *et al.* (1987*ab*, 1989), one needs to have a general view of what a scientific theory is and what role it has to play in science, particularly in experimental science. In this introductory chapter, therefore, I plan to discuss these questions, with the hope of providing a context in which the claims of metabolic control theory as a legitimate theory can be discussed. Elsewhere (Cornish-Bowden, 1989) I have discussed the criticisms of metabolic control theory made by Savageau and his colleagues (Savageau *et al.*, 1987*ab*) in a specific way, and will not repeat the arguments in detail here; rather I shall use metabolic control as a context for discussing in general what a theory ought to be and what it should offer.

Does Biochemistry need Theories?

Biochemists have long been suspicious of theory, doubting whether it can substitute for serious experimentation. And, of course, it cannot; no piece of biochemical knowledge has ever been produced by pure thought uncontaminated by any information about the real world. Atkinson emphasizes this point in the Prologue and in Chapter 36 of this book, and Crick (1989) has recently commented that "it is virtually impossible for a theorist, by

Athel Cornish-Bowden ● Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

thought alone, to arrive at the correct solution to a set of biological problems. Because they have evolved by natural selection, the mechanisms involved are usually too accidental and too intricate."

Equally, however, one may doubt whether any significant advances in our understanding of metabolic systems have come from pure experimentation uncontaminated by a theoretical framework within which the observations can be interpreted. Indeed, it is likely that the second misconception about how knowledge advances is more damaging than the first, because the theoreticians who work in complete isolation from any experimental information are few in number and they consume few resources, whereas experimentalists who work with a completely open (perhaps empty) mind are distressingly common and they do consume resources.

Nonetheless, it does not follow than just any theory is useful, and one needs to be able to recognize the characteristics of a model that allow it to provide a framework for interpreting nature. If it is too general, so that it can accommodate absolutely any new piece of knowledge without requiring any modification, then it cannot be falsified and is not scientific according to the philosophy of science associated with Popper (1959). On the other hand, if it is too rigid, so that it cannot accommodate even a slight change to the body of experimental facts without major revision, it is hardly useful either. We must also allow some degree of fuzziness in the criteria for falsification: if a few facts are not exactly consistent with our basic theory, we should certainly note them as matters that need to be properly explained eventually, but we should not feel obliged to discard the theory immediately if no better one is available, and if the facts that cannot be accommodated represent rare or special circumstances.

The discovery of fructose 2,6-bisphosphate by van Schaftingen & Hers (1980) illustrates the point very well. Before this regulator of various enzymes was discovered there were many facts about the control of phosphofructokinase (in particular) that were inconsistent with the ideas of the control of glycolysis that had been accumulating for many years; as a result all extant theories were not merely falsifiable but were, in fact, falsified. This falsification could, of course, be taken as a pointer to the existence of an unknown regulator, but on an extreme interpretation it could have been taken to require the whole of current ideas of glycolysis and its regulation to be rejected. If they had been rejected, the result would hardly have been to clear the way for the rapid discovery of fructose 2,6-bisphosphate but rather to have left the whole field of research without any context in which experiments could be planned and analysed. With the hindsight of our current knowledge of fructose 2,6-bisphosphate we can argue that the earlier ideas were not so much wrong as incomplete. The proper response to a falsifying experiment, therefore, is neither to reject the theory nor to ignore the experiment; rather we should retain the theory as a basis for discussion until a better one becomes available, but we should simultaneously regard the conflicting experiment as something that must in due course be accommodated. The role of falsifiability is not so much to destroy theories at a moment's notice as to impose on the theoretician the need to consider which types of result would require the theory to be discarded and which would merely need to be noted for attention later.

One may note in passing the view often ascribed to Popper (1959) that Darwin's theory

of evolution by natural selection is not falsifiable and hence not scientific. Although this criticism is not directly related to metabolic control, we cannot simply ignore it, because it implies that anyone committed to the theory of evolution by natural selection (as are virtually all biologists, with, of course, some reservations about points of detail) has no choice but to reject Popper's view of what is scientific. This would be unfortunate, both because Popper has withdrawn the criticism (something that appears to be known by few of the people who quote it), and because it is anyway based on a failure to appreciate the kinds of experiments that one can easily do that would falsify the theory of evolution if it were false, as discussed, for example by Penny *et al.*(1982). Suffice it to note here that one can accept the importance of falsifiability as a criterion of what is scientific without having to reject the theory of evolution.

However, one can accept falsifiability as *a* criterion without regarding it as the only possible criterion, and without agreeing that theories that are not falsifiable have no value in science. Suppose that the theory of evolution were indeed unfalsifiable: would it then have no value in biology? Many of us would still agree with Dobzhansky (1973) that "nothing in biology makes sense except in the light of evolution", and if so it is surely clear that a theory that allows us to rationalize a huge body of otherwise disorganized knowledge is of great value even if it makes no predictions whatsoever. For a full and masterly discussion of this in relation to evolution one may refer to Dobzhansky *et al.* (1977). This is perhaps no more than a specific instance of the fact that insofar as working scientists are conscious of philosophy at all in their daily work, the philosophy that guides them is that of William of Ockham, not that of Popper: they will prefer the theory that introduces the fewest number of assumptions, regardless of whether it is falsifiable.

Terminology

The ideas of Kacser & Burns (1973), Heinrich & Rapoport (1974), Savageau (1969*ab*, 1970, 1976), and Crabtree & Newsholme (1987), not to mention theories of enzyme cooperativity [reviewed in Ricard & Cornish-Bowden (1987)] and a host of *ad hoc* proposals to do with the control of specific systems and pathways, can all be generally categorized as theories of metabolic control. Why then should one limit the term "metabolic control theory" to the body of theory developed from the papers of Kacser & Burns (1973) and Heinrich & Rapoport (1974), which is introduced in this book by Porteous, in Chapter 3? The primary justification is one of convenience: this is the way in which the term has come to be used by many workers in the field, including most of the contributors to this volume, and it does little harm provided that one remembers that it is not the only conceivable theory to have a claim to the name. Similarly, it is convenient to use the term "biochemical systems theory" to refer to the ideas of Savageau (1969*ab*, 1970, 1976; see also Chapters 4 and 5 in this book, by Savageau and Voit respectively), and I shall follow this usage in this chapter, although, again, there are other theories that could conceivably be known by the same name.

One may note in other chapters of this book a growing tendency to prefer the term "metabolic control *analysis*", on the basis that what it (and, equally, biochemical systems theory) offer is not so much a theory of how real systems behave as a mathematical method for analysing how they behave. This is an important distinction, but it seems to me to be an exaggeration to argue that there is no theoretical content in the two approaches.

Falsifiability and Falsification

The supposed unfalsifiability of the theory of evolution is equally characteristic of cosmology, and can be answered in much the same way. Curiously, however, the cosmologists have not had to suffer to the same extent the suggestion that they are working in metaphysics rather than science. The concerns of the "creation scientists" with evolution can hardly provide the whole explanation for this discrimination, because cosmology is no less important in theological arguments than evolution, and, indeed, cosmology deals with an even longer time scale than evolutionary biology. Perhaps it is simply a manifestation of the disdain that some physical scientists have for biologists, so that people feel it is safer, and less obviously absurd, to accuse biologists of working outside science, than to make the same accusation of physicists.

Even if falsifiability — the capability of being tested — is essential in science, actual falsification plays a much greater role in engineering than in science. where it is a crucial test of the validity of a model. To design a bridge or an aircraft wing, it is useful but not by any means essential to have a good understanding of the stresses that will cause it to fail. What is essential in this case is reliability, not comprehensibility: an equation that accurately predicts the behaviour over the whole range of conditions of use is far more useful, even if it is a purely empirical equation with no theoretical base at all, than an equation that faithfully reflects the best available theory but deviates appreciably from the actual behaviour. Of course, one always hopes that a good theory will lead to better models, and this is, of course, the belief that sustains many academic attempts at rational drug design. In the words of Leonid Brezhnev [as quoted by Rich (1977)], "there is nothing more practical than a good theory".

To appreciate the difference between modelling and understanding, between engineering and science, one may compare different equations for expressing the behaviour of enzymes that deviate from Michaelis-Menten kinetics. On the one hand the Hill equation (Hill, 1910):

$$v = Vx^h/(K + x^h) \tag{1}$$

in which v is the rate of reaction at a ligand (substrate) concentration x and V, K and h are parameters, is simple to write, easy to use, and gives an excellent prediction of the actual behaviour of many enzymes over the whole range of interest. The equation of Monod *et al.* (1965):

$$v/V = \frac{[Lc(1 + cx/K_R)^3 + (1 + cx/K_R)^3]x/K_R}{L(1 + cx/K_R)^4 + (1 + cx/K_R)^4} \tag{2}$$

in which v and x have the same meanings as in the Hill equation and V, L, c and K_R are parameters, is much more complex in appearance, and it contains four adjustable parameters instead of three. Yet it is hardly better than the Hill equation at fitting real data, even at best, and sometimes gives a very poor fit to data that fit the Hill equation well. In what circumstances, then, might we nonetheless prefer to use the equation of Monod *et al.*? From the point of view of modelling, there are very few such circumstances: we should clearly prefer the simpler equation if it fits real data just as well or better. If our objective is to understand the physical basis of the deviations from Michaelis-Menten kinetics, however, we cannot achieve this understanding on the basis of an empirical equation that is not derived from a physical model. We must then use equations derived from our hypotheses about the underlying mechanism. If these fail to fit, as they well may, we must search for a physical model that gives equations that do fit; we cannot simply retreat to an equation such as the Hill equation that tells us nothing about the mechanism.

The Hill equation is also useful to illustrate the different *levels* at which one may seek to understand a phenomenon. One can study metabolic control not only at the level of enzyme mechanisms, but also at the level of interplay between all of the enzymes in a pathway. In the latter case, we may reasonably decide to take the behaviour of each individual enzyme as given, and study only the effects due to simultaneous action of several or many enzymes. For this purpose the Hill equation is very useful, normally more useful than mechanism-based alternatives.

Objectives: Understanding, or Prediction

In the effort to understand the kinetics of multi-enzyme systems, there have been two fundamentally different approaches that have differed not so much in the detailed assumptions that they have made (though these do differ as well, within both approaches). One group of workers, who include not only the believers in rate-limiting steps, metabolic bottlenecks, key enzymes, etc., but also the adherents of metabolic control theory, are primarily concerned with understanding what happens in the living cell. If their understanding allows them to become effective modellers of metabolism in the computer, or to design efficient fermentation systems, that is an attractive bonus but it is not the primary objective. Others, however, have been interested in setting up computer models of metabolic systems or developing equations that allow them to predict how complex systems will behave in different conditions. This approach was pioneered by Garfinkel & Hess (1964) many years ago in the first computer models of metabolism, and has been followed in the development of biochemical systems theory and in other systems (e.g. Liao & Lightfoot, 1987) that seek to define the behaviour of large complex systems over a finite range of conditions.

It is my impression that some of the controversies that have arisen over metabolic control theory and biochemical systems theory have their origins in failure to appreciate that the objectives are not identical. If each theory is judged by the extent to which it meets the objectives of the other one cannot be surprised if each turns out to be inferior to the other. It

may be, of course, that I have misinterpreted the aims of the originators of metabolic control theory and biochemical systems theory, but it seems to me that the former are primarily concerned with understanding why real systems behave as they do, whereas the latter are more interested in developing models that accurately reproduce the behaviour of real systems.

Originality and Triviality in Theory

An aspect of theory that seems to have been discussed hardly at all in the biological literature is the question of what constitutes originality. This is hardly worth discussing at all in relation to experimental biology because in some sense almost any research can be regarded as original, even if consists of no more than repeating on *Drosophila pseudoobscura* a series of experiments previously done on *Drosophila melanogaster*. If one believes that research is uncontaminated by any preconceptions, i.e. any theoretical base, one can hardly say that this kind of experimentation is any less original than any other.

However, when one comes to consider theory, especially theory that has a strong mathematical content, one runs the risk of going to the opposite extreme and denying that any analysis of a system at all can be original. The model of Monod *et al.* for cooperativity in a tetrameric protein [shown in eqn. (2) above] is mathematically no more than a special case of the Adair equation for a tetramer:

$$v/V = \frac{K_1x + 3K_1K_2x^2 + 3K_1K_2K_3x^3 + K_1K_2K_3K_4x^4}{1 + 4K_1x + 6K_1K_2x^2 + 4K_1K_2K_3x^3 + K_1K_2K_3K_4x^4} \tag{3}$$

in which $K_1 \ldots K_4$ are constants and the other symbols have the same significance as in eqn. (2). That eqn. (2) is a special case of eqn. (3) may be seen by defining the new parameters as follows:

$$K_1 = \frac{1 + Lc}{K_R(1 + L)} \tag{4}$$

$$K_2 = \frac{1 + Lc^2}{K_R(1 + Lc)} \tag{5}$$

$$K_3 = \frac{1 + Lc^3}{K_R(1 + Lc^2)} \tag{6}$$

$$K_4 = \frac{1 + Lc^4}{K_R(1 + Lc^3)} \tag{7}$$

The only physical restriction on the values allowed to the parameters in these equations is that they must all be positive and finite. But it is evident from eqns. (4-7) that if this is true of L and c then it must also be true of the Adair constants $K_1 \ldots K_4$. Thus eqn (2) predicts nothing that is not also predicted by eqn. (3), or, in words, the model of Monod *et al.* (1965) adds nothing to what was already contained in the model of Adair (1925*ab*) published 40

years earlier. Equally the model of Koshland *et al.* (1966) adds nothing to the model of Adair, in common with other models of cooperativity in oligomeric proteins published in the 1960s and 1970s.

Must we conclude, therefore, that these models are simply rediscoveries of the work of Adair and that they have no right to be regarded as original contributions to knowledge? If we must, is there any reason to regard Adair as the source of all theory about protein behaviour? After all, eqn. (3) is no more than a rational function of a kind thoroughly studied in earlier centuries. In a sense, all mathematics is wholly contained within its set of axioms, and thus no mathematical research, and no theory that is in essence no more than applied mathematics, introduces anything new. If I correctly understand Gross (1988; quoting Yang, 1977), many mathematicians do not even regard the introduction of novel axioms as original work: they see themselves as doing no more than giving expression to "natural" ideas that were there all the time waiting to be revealed.

It might seem that we have reached a stage in which the whole idea of originality has neither point nor meaning. Yet clearly scientists do attach both meaning and point to the idea of originality, and my *reductio ad absurdum* does not do justice to the way originality is actually perceived. The papers of Monod *et al.* (1965) and of Koshland *et al.* (1966), and especially the arguments about which of them embodied the "truth", did more to stimulate experimental research in the 1960s and 1970s than even the most careful reading of Adair's papers, let alone an 18th century treatise on algebra, could have done; we can hardly doubt, therefore, that experimentalists saw something original and interesting in these papers.

I believe that the search for more general ways of expressing existing models, well illustrated in protein chemistry by numerous efforts (mostly now consigned to a well merited oblivion) to develop models of cooperativity that include the models of Monod *et al.* (1965) and Koshland *et al.* (1966) as special cases, rarely provides the road to a better understanding. Probably there are exceptions, but I cannot immediately recall any.

I suggest, on the contrary, that we ought not to ask what Monod *et al.* (1965) added to the model of Adair, but what they subtracted from it: in other words, what did they remove from the universe of conceivable ways in which a tetrameric protein could behave within the constraints imposed by thermodynamics, in order to arrive at a postulated universe of ways in which real tetrameric proteins do behave? If this is accepted as a valid criterion of originality, it is obvious that we cannot dismiss a theory on the grounds that it is a special case of a theory that was developed earlier. On the contrary, we have to interpret original theory as theory that reveals novel special cases that are sufficiently interesting to stimulate efforts to build on them or falsify them. Indeed, it seems to me that consideration of originality leads inevitably back to falsifiability: if a theory is so general that it is hard to conceive of a way of falsifying it, then it is hardly within the domain of science. To make it more scientific, therefore, one must subtract from it, not add to it; move towards the more special, not towards the more general.

As well as originality, we commonly demand of a contribution to knowledge that it should not be trivial or obvious, but in practice this is very difficult, even for the author of the contribution, to judge. Slater (1988) has recently described his thoughts when he first conceived of the chemical hypothesis of energy transduction by analogy with the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase. Was the idea worth proceeding

with, or was it too trivial even to report? With the perspective of thirty years we might well categorize this hypothesis as mistaken, or even as misconceived, but hardly as trivial. My own view is that apparent triviality is one of the possible criteria for judging the importance of a new idea, and that many of the most important advances have seemed so trivial as to be hardly worth saying, once, of course, they have been said. Certainly, if I may inject a personal note, the advances made by others that have impressed me most have not been the ones that seemed very difficult to understand, let alone difficult to have made myself, but the ones that have provoked the immediate response "why didn't I think of that?". For example, at one time I was interested in coupled enzyme reactions in the context of assay systems. In publishing our work (Storer & Cornish-Bowden, 1974) I was aware that some of the same considerations applied to multi-enzyme metabolic processes, but even after the opportunity to reflect on this for seven years, it still came as a revelation (Easterby, 1981) to learn that a fairly simple extension to the calculation of the time required for a two-enzyme system to reach 99% of its steady-state flux could lead to a highly useful way of thinking about transition times for cellular processes. [More recent work in the area of transition times is described by Easterby in Chapter 23.]

Of course, sometimes ideas seem to be trivial and obvious because they are indeed trivial and obvious, and one should certainly ask the question; it would be absurd to claim that apparent triviality is an infallible indicator of importance. However, before dismissing an idea as being an obvious extension of existing knowledge, one should enquire why, if its usefulness is not in question, it was not previously pointed out; why its importance was not recognized. In this connection one can hardly do better than to read Feynman's entertaining discussion of the proposition that "mathematicians only prove things that are obvious" (Feynman, 1985).

Priority in Scientific Discovery

Much of the bitterness that accompanies arguments about scientific theories has its basis in fears that one's discoveries will be attributed to others. One cannot simply dismiss priority disputes as being irrelevant to the march of science, because one can hardly doubt that hopes for recognition and fame provide a powerful motivation for undertaking research in the first place. One can, however, insist that priority disputes are primarily of interest to those involved in them, and can try to avoid allowing time at conferences and pages of journals to be wasted on them.

Those who feel that their contributions have been set aside or ignored are usually willing to imply base motives to their opponents, but in reality forgetfulness, failure to read the literature properly and other examples of inefficiency rather than deliberate malice are often to blame. Moreover, it is only too easy, when one makes a novel observation but then sees it published by another group before one has written it up, to convince oneself that this publication never happened or that it only hinted at the observation. I shall give an example from my own experience, but I do not believe that I am unique. Some time in 1971, I became convinced that the kinetic data of Parry & Walker (1967) for hexokinase D ("gluco-

kinase") from rat liver indicated that this enzyme displayed positive cooperativity with respect to glucose. Although the original authors had not reached the same conclusion, it seemed only mildly interesting in 1971 because there was not at that time any reason to think that it was a monomeric enzyme. Thus it was not until several years later that a paper from our laboratory appeared that described the cooperativity (Storer & Cornish-Bowden, 1976), by which time Niemeyer *et al.* (1975) had reported the same observation; we had also become convinced that the enzyme was monomeric, so that its cooperativity was much less ordinary than it had seemed. After 13 years I cannot remember exactly what was in our minds when we wrote the 1976 paper: I do not believe that we deliberately set out to denigrate the observations of Niemeyer *et al.*, but we nonetheless referred to a report that "glucokinase exhibits a sigmoidal saturation function" (a point made several times in their paper) as simply one of three examples of "slight deviations from Michaelis-Menten kinetics observed by earlier workers". Partly, no doubt, we were anxious to avoid encouraging the referees to believe that we were doing no more than confirming a known result, but I think we were also unready to admit to ourselves, let alone to the world, that something we had believed since 1971 had been published by a group in Chile before we were ready to publish it ourselves. That no lasting bitterness was created by this example, may be seen from the fact that the second author of the 1975 paper is also the co-editor of this book, not to mention a closer and more long-term relationship.

Implications for Metabolic Control Theory

Many of those now interested in metabolic control theory "know" without wasting their time with arguments of the sort that I have been discussing that they are dealing with an important and original contribution to metabolic control. Moreover, I have deliberately refrained here from relating the arguments too directly to metabolic control theory, not with the idea of making them more abstruse and boring, but with the object of avoiding for the moment a controversy that is raised by more expert voices elsewhere in this symposium, and with the object also of showing that there is nothing special about the claims of metabolic control theory: if we apply the same criteria of originality that would apply to theories in other branches of knowledge then we will, I believe, conclude that metabolic control theory is a valid theory.

There are, however, other questions that need to be answered satisfactorily before accepting that metabolic control theory is *the* theory that should underlie discussion of metabolic control. The experimentalist will certainly want to know whether it makes assumptions about the way metabolic systems behave that correspond with reality: in the Prologue of this book Atkinson suggests that it is found wanting in this respect, but his arguments are questioned by Fell and Sauro later in the Prologue. If it does make reasonable assumptions, does it set out a protocol for study real systems that will allow experiments to give a better understanding of how they are controlled in the cell? Regardless of one's answers to these questions, one should also ask them in relation to alternative theories to explain metabolic control: do biochemical systems theory, or the traditional ideas of rate-

limiting steps and key enzymes, or the modified version of these ideas advocated by Crabtree & Newsholme (1987), or any other theory, provide better or more satisfying answers?

REFERENCES

Adair, G. S. (1925*a*) *J. Biol. Chem. 63*, 529-545
Adair, G. S. (1925*b*) *Proc. Roy. Soc., Ser. A 109*, 292-300
Cornish-Bowden, A. (1989) *J. Theor. Biol. 136*, 365-377
Crabtree, B. & Newsholme, E. A. (1987) *Biochem. J. 247*, 113-120
Crick, F. (1989) *What Mad Pursuit: a Personal View of Scientific Discovery*, pp. 108-115, Weidenfeld and Nicolson, London
Dobzhansky, Th. (1973) *Amer. Sci. Teacher 35*, 125-129
Dobzhansky, Th., Ayala, F. J., Stebbins, G. L. & Valentine, J. W. (1977) *Evolution*, pp. 474-516, Freeman, San Francisco
Easterby, J. S. (1981) *Biochem. J. 199*, 155-161
Feynman, R. P. (1985) *Surely you're joking, Mr. Feynman!*, pp. 84-87, Unwin Paperbacks, London
Garfinkel, D. & Hess, B. (1964) *J. Biol. Chem. 239*, 971-983
Gross, D. J. (1988) *Proc. Natl. Acad. Sci. USA 85*, 8371-8375
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42*, 89-102
Higgins, J. (1963) *Ann. N. Y. Acad. Sci. 108*, 305-321
Higgins, J. (1965) in *Control of Energy Metabolism* (Chance, B., Estabrook, R. K. & Williamson, J. R., eds.), pp. 13-46, Academic Press, New York
Hill, A. V. (1910) *J. Physiol. (Lond.) 40*, *iv-vii*
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27*, 65-104
Koshland, D. E., Jr., Némethy G.& Filmer, D. (1966) *Biochemistry 5*, 365-385
Liao, J. C. & Lightfoot, E. N., Jr (1987) *J. Theor. Biol. 126*, 253-273
Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Molec. Biol. 12*, 88-118
Niemeyer, H., Cárdenas, M. L., Rabajille, E., Ureta, T., Clark-Turri, L. & Peñaranda, J. (1975) *Enzyme 20*, 321-333
Parry, M. J. & Walker, D. G. (1967) *Biochem. J. 105*, 473-482
Penny, D., Foulds, L. R. & Hendy, M. D. (1982) *Nature 297*, 197-200
Popper, K. R. (1959) *The Logic of Scientific Discovery*. Hutchinson, London (translated from *Logik der Forschung*, Vienna, 1934)
Ricard, J. & Cornish-Bowden, A. (1987) *Eur. J. Biochem. 166*, 255-272
Rich, V. (1977) *Nature 270*, 470-471
Savageau, M. A. (1969*a*) *J. Theor. Biol. 25*, 365-369
Savageau, M. A. (1969*b*) *J. Theor. Biol. 25*, 370-379
Savageau, M. A. (1970) *J. Theor. Biol. 26*, 215-226
Savageau, M. A. (1976) *Biochemical Systems Analysis: a Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts
Savageau, M. A., Voit, E. O. & Irvine, D. H. (1987*a*) *Math. Biosci. 87*, 127-145
Savageau, M. A., Voit, E. O. & Irvine, D. H. (1987*b*) *Math. Biosci. 87*, 147-169
Slater, E. C. (1988) in *The Roots of Modern Biochemistry* (Kleinkauf, H., von Döhren, H. & Jaenicke, L., eds.) pp. 625-630, de Gruyter, Berlin
Storer, A. C. & Cornish-Bowden, A. (1974) *Biochem. J. 141*, 205-209
Storer, A. C. & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 7-14
van Schaftingen, E. & Hers, H.-G. (1980) *Biochem. Biophys. Res. Commun. 96*, 1524-1531
Yang, C. N. (1977) *Ann. N.Y. Acad. Sci. 294*, 86

Chapter 2

History and Original Thoughts on the Control Theoretic Approach

JOSEPH HIGGINS

I HAVE BEEN asked to present some historical background on the early theory of metabolic control; I am pleased to do so. I guess I was asked to do this because to some, at least, I am considered a grandfather of the field. I am not sure how I came to be a grandfather without ever being a father. Yet let that be, for I accept the accolade with sincere appreciation even though I think it partly, if not wholly, apocryphal. But if there are grandfathers, there must also be great-grandfathers and even great-great-grandfathers lurking in the woods. So with no small concern about the validity of such terms, I shall nevertheless address this history in those terms. Hopefully some grandchildren will be reading too. But let me note at the outset that I am not a scientific historian and that I present this history from a rather personal view.

The Great-grandfather and the Development of Reflection Coefficients

To my mind at least, one of those great-grandfathers is Britton Chance, my mentor. His encouragement, guidance and intellectual transfer were a major part of my scientific development and thinking. But his contributions to the field of metabolic regulation, both direct and indirect, go far beyond me. They can be found in the use of computer models to represent and study metabolic pathways and in the language we use to describe and think about these systems in terms of feedback and control properties (a semi-electronic language). His formal training was in Electrical Engineering (BA) and in Physical Chemistry (PhD) at the University of Pennsylvania. His thesis is a classic paper (Chance, 1943), for in that paper he set the tone for years to come, not only for his own research, but for that of many others, including myself. In his thesis, Chance demonstrated, using the peroxidase reaction and spectrographic methods, that the Michaelis-Menten enzyme-substrate complex really existed; he made a further application of rapid reaction techniques in biochemistry, and he used the MIT (Massachusetts Institute of Technology) *mechanical* differential analyser to solve

Joseph Higgins ● Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

the non-linear differential equations of the reactions and to compare the experimental and theoretical results, and from those studies he derived a simple method for determining one of the rate constants. Although no one of these techniques was entirely new at the time, his paper presents the first serious integration of experimental and theoretical studies with the use of an analogue computer.

His postdoctoral studies were with F. J. W. Roughton and H. Theorell, where he further developed the rapid flow methods and their application. But more important to the field of metabolic control, he spent the war years at the MIT Radiation Laboratories where many of the principles of electronic control theory were developed (Chance *et al.*, 1949).

In 1948, he became the director of the Elridge Reeves Johnson Foundation (the JF), which was affiliated with the University of Pennsylvania. Among the many rare and remarkable things that Chance has done, he chose to hire scientifically oriented high school graduates as potential technicians. Scholarships were very few in those days and this provided those students with a means to attend college at night at the reduced cost offered by the University for its employees. I was one of those students.

It was a time of few free hours — going to work during the day and to school at night (which included Saturdays as well). Yet it was the most remarkable of times. As undergraduates, we mingled with graduate students, post-doctoral fellows and junior and senior scientists. There were usually lunch-time seminars, often by visiting scientists, to the point that many who were at the JF during the 1950s and 1960s still salivate when they attend seminars. Chance brought in not only pure experimentalists, but physicists and mathematicians as well. Although his own interest is primarily experimental, he was (and is) uncommon in promoting and encouraging the development and application of mathematics to the fields of biochemistry and biophysics. And by generously giving credit when due, he promoted our scientific careers as well.

My formal studies were in physics, with a minor in mathematics. But the JF atmosphere was such that anyone could learn as much or more informally. Chance initiated the development of the first fully electronic analogue computer in 1949. I arrived in June 1949 and started as an electronics technician and gradually learned the theory of electronic design (Chance *et al.*, 1951). After its completion, I became the computer operator. Chance would set particular problems for study, but allowed all of us a rather free rein in the actual study and analysis of the problem. In addition, there was ample time to play with the computer, study the results and develop the mathematics. The specific studies began with the peroxidase system (Chance & Higgins, 1952) and later a mass action model of oxidative phosphorylation. I obtained my BA in 1954 and spent a year at Harvard studying physics. I returned to the JF in 1955 and was joined by another graduate student, William Holmes, in the computer and mathematical studies of these systems (Chance *et al.*, 1955). At Chance's suggestion we studied and developed the crossover theorem (Chance *et al.*, 1958), one of the earliest tools for analysing the regulation of oxidative-phosphorylation.

At about the same time, Chance initiated the development of a digital computer program for the simulation of metabolic pathways on the recently developed Univac digital computer, which was a vacuum tube computer that had to be programmed in machine language as no compilers had yet been developed (Garfinkel *et al.*, 1961). One of the earliest applications

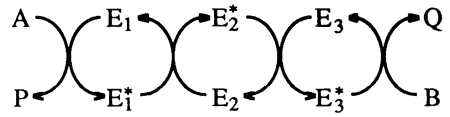
was the study of metabolic control between glycolysis and oxidative phosphorylation using a simplified model of 22 metabolic reactions (Chance *et al.*, 1959).

My formal studies continued in physics where I concentrated on thermodynamics and statistical mechanics. But my thesis research was based on computer and mathematical studies of certain types of non-enzymatic and enzymatic sequences. In particular:

$$\text{Type 0:} \quad [S_0] \Leftrightarrow S_1 \Leftrightarrow S_2 \Leftrightarrow S_3 \rightarrow$$

$$\text{Type I:} \quad E + S \Leftrightarrow ES_1 \Leftrightarrow ES_2 \rightarrow E + P$$

Type II:



where the last is a model for oxidative phosphorylation, and a further generalization of the type II sequence which allowed additional intermediates in each cycle. On the computer, I studied the kinetics of the intermediates and the substrate (which was consumed). For the type I system there is an initial transient state in the formation of ES complexes but this is followed by a slowly changing "steady state" region which very nearly obeys the ordinary steady state relationships between substrate and intermediate concentrations.

Based on the proportionality of the ES complexes, a closer analysis led me to consider the relative change in the ES complexes (in particular $\Delta p_i/p_i$). And as I was dealing with small changes, I investigated the small relative changes in the various complexes, the substrate and the rate. My view at the time was that the relative changes in any particular variable was related to, or as I put it, *reflected by* the relative changes in some other variable. Thus, if x and y are the variables, then the change in y relative to the change in x is given by the following expression:

$$\frac{\Delta y}{y} = x^{\Delta}_{Ry} \cdot \frac{\Delta x}{x}$$

where x^{Δ}_{Ry} was defined as the finite reflection coefficient. In the limit as Δx and Δy become small we replace the Δ by differentials and write

$$x_{Ry} = \frac{dy/y}{dx/x} = \frac{d\ln y}{d\ln x} = \frac{x}{y} \cdot \frac{dy}{dx} = \lim_{\%\to 0} \frac{\%\text{ change in } y}{\%\text{ change in } x}$$

and where dx and dy can be replaced by their time derivatives dx/dt and dy/dt.

The reflection coefficient was clearly closely related to the ordinary derivative (dy/dx), and it was easy to realize that it satisfied many of the same properties. Thus

$$\frac{dy}{dx} = \frac{1}{\frac{dx}{dy}} \quad \text{and} \quad x_{Ry} = 1/y_{Rx}$$

$$\frac{dz}{dx} = \frac{dz}{dy} \cdot \frac{dy}{dx} \quad \text{and} \quad x_{Rz} = x_{Ry} \cdot x_{Rz}$$

with obvious extensions to other rules of the differential and partial differential calculus. It is important to note that the definition of the reflection coefficient was generic, just as the ordinary derivatives, and could likewise be applied to any two variables. There are also differences which attracted me to the study of relection coefficients. For one thing, the reflection coefficient is dimensionless, regardless of the different dimensions of the variables; the ordinary derivative is not. In retrospect, I should have chosen some other term such as "reflective derivative" to emphasize that it is a derivative. But then again, Newton had his fluxions.

A second feature is that the reflection coefficient simplified the results for many functions which arise in the analysis of biochemical reactions. Thus, for a type I system as follows:

$$S + E \Leftrightarrow ES_1 \Leftrightarrow E_2 \rightarrow E + P \tag{1}$$

If s, q, p_1 and p_2 represent the concentrations of S, E (free), ES_1 and E_2 respectively and $e = q + p_1 + p_2$ then the rate is given by the following expression:

$$\frac{Vs}{s + K_m} \tag{2}$$

The ordinary derivative yields

$$\frac{dv}{ds} = \frac{VK_m}{(s + K_m)^2} \quad \text{while} \quad s_{Rv} = \frac{K_m}{s + K_m} \tag{3}$$

so that the reflection coefficient yields simpler results as to the number of parameters and the degree of equation.

Further, at the time I was particularly interested in finding ways to use the experimental data to determine the chemical mechanism involved, or at least to eliminate the number of possibilities. And in that regard, the reflection coefficient appeared particularly useful. For the type I system [eqn. (3)], one finds that

$$s_{Rv} = \frac{q}{e} = \frac{e - p_1 - p_2}{e}, \; p_{1Rp_2} = 1 \text{ and } v_{Rp} = 1 \tag{4}$$

Thus if the concentrations and rates can be measured then, at least in principle, these equations can be tested. These equations tend to be unique to the type I system; the type II system yields quite different and easily distinguishable results. The results for a simple sequence of non-enzymatic reaction, the type 0 system,

$$[S_0] \Leftrightarrow S_1 \Leftrightarrow S_2 \rightarrow \tag{5}$$

gives

$$s_{0Rv} = 1 \quad \text{and} \quad s_{iRs_j} = 1 \quad \text{for all } i \text{ and } j \tag{6}$$

where s_i represents the concentration of S_i.

It should also be noted that the relative changes are a form of differentials and thus the equations giving values of the reflection coefficients represent a set of differential equations. They can be integrated. The eqns. (6), with the additional condition that $e = q+p_1+p_2$, can be integrated to yield the original steady-state equation. The V and K_m come in as the constants of integration. It would of course be useless to rederive the equations we started with but in principle, at least, and in actual fact, reflection coefficient relations can be established by experiment or indirect theories. Those relations can be integrated to yield direct relations between the variables.

The complete development of reflection coefficients as well as other theorems are published in my thesis (Higgins, 1959) and many of these results are reported in later publications (Higgins, 1961, 1963).

The Development of Control Theory

Lurking in the woods here are not only great-grandfathers but great-great-grandfathers. First, I should note that I am not a great believer in the idea that the so-called "new theory" of control is the end-all for either the experimentalist or the theoretician. Second, I have a great respect for the so-called "old theory". So I shall begin there.

Let me begin by quoting Burton (1937): "The principle of the 'master reaction' is that the 'slowest' of the reactions in a process which involves a chain of reactions determines the rate of the whole. This generalization was made by Blackman (1905) who stated it as an axiom, evidently believing it to be self evident, that when a process is conditioned as to its rapidity by a number of separate factors, the rate of the process is limited by the pace of the slowest factor. The principle was developed by Putter (1914) and should be known as the Blackman-Putter principle". [Speaking of fatherly relations, at the time of these papers, Burton was at the Johnson Foundation, and Chance was well aware of his work (Chance *et al.*, 1962)]

But Burton goes on to analyse the experiments of Crozier (1924). Crozier had concluded the existence of master reactions by the temperature dependence of various *in vivo* processes over a range of species. He found that many species had the same activation energy for similar reactions and concluded that they were probably governed by the same master reaction. Burton (1937) argued against Crozier's results and even the existence of any "Master Reaction" on theoretical grounds. Burton concludes that "True master reactions may well be in operation, but there is at present no means of proving their existence".

However, in a later paper, Burton (1939) analyses a sequence of three isomeric reactions (a type 0 system). He discusses the conditions needed for a steady state (stationary state) to be achieved; i.e. the necessity of a source and sink. He does not actually solve the steady state rate equations but notes that by a complicated proof it can be shown that the rate is less

dependent on any particular rate constant as that rate constant becomes larger. And that the converse is also true; the lower the value of a rate constant and "hence the 'slower' the reaction it governs, the greater its influence on the whole system, is connected with the well-known 'Principle of the Master Reaction'". He discusses this only for the simplest system. It should be noted that he uses a real "water-bottle" flow model as an analogue; probably, the first use of an "analogue computer" in the field of biochemistry. In any case, he seems at that point to have accepted the principle of a Master Reaction. But the quotation marks he puts around the "slower" leaves no doubt as to his questions about what that means. Certainly, he must be acknowledged as one of the early investigators in the field of metabolic control.

A complete solution for the rate of a fully reversible system of pseudo-first order reactions was developed by Christiansen (1935) and Hammett (1937). Morales (1947) includes a single, irreversible Michaelis-Menten enzyme in such a sequence and argues against Burton's (1937) critique of the Master-Reaction principle. He shows that a master reaction can exist under certain conditions. I note that Burton (1939) seems already to have changed his mind or at least was vacillating.

I think it is fair to say that the theoreticians of that time were in something of a quandary and indecisive as to whether a Master Reaction could exist under realistic conditions. All the simplified theoretical models which yielded a Master Reaction seemed to require that reaction to be an irreversible step in the sequence (and without any feedback such as product inhibition). In retrospect, that requirement was more of a necessity in solving the equations and has little to do with the existence, or lack thereof, of a Master Reaction. In addition, the link between fast and slow reactions and Master-Reactions was theoretically unclear as was the definition of a fast or slow reaction itself.

But whatever the quandaries the theoreticians were having, the experimentalists were setting the concepts into stone, or so it appears even as judged by the arguments of this meeting and certainly by numerous textbooks of that time and now. Being at the JF, I had ample opportunity to learn the experimentalists' beliefs at first hand. Perhaps I overstate, but the concept was that the first reaction of any sequence or at the beginning of a branch point was an irreversible reaction (unaffected by its products). This was the so-called "committed" step and it was also the "rate-limiting" reaction. It was also the slowest reaction (whatever that meant). Krebs had an additional point concerning the near-equilibrium reactions which he and his students had spent much time investigating as an experimental analytical tool; he felt that such reactions were necessarily fast and therefore could not be rate controlling.

I became involved in these problems in 1963, after I returned from my post-doctoral studies. The experimentalists had discovered oscillating reactions that were linked to glycolysis (Chance *et al.*, 1964), and I became involved with the feedback and control properties of that system (Higgins, 1964, 1967). I should note that I had not preconceived notions of metabolic control; I learned the experimentalists view at first hand (I had not read the papers I cited above regarding prior studies of the master reaction at that time — in fact, I did not read them until I prepared this paper). Perhaps I was lucky to approach the problem with an open mind. But, the experimentalist's view was quite clear — although glucose utilization begins with hexokinase (a rate-limiting reaction), the products (glucose 6-phosphate and fructose 6-phosphate) can go to glycogen or into the pentose-phosphate shunt. The first

committed step of glycolysis (to pyruvate) begins with phosphofructokinase, which is also rate limiting.

I did not set out to destroy that view. Rather, I wanted to examine the concepts and to find a way to pull them together. Although I understood what experimentalists meant by a "rate-limiting reaction", it annoyed me no end since in a stationary state all the rates are equal. I also wanted to understand more precisely the definition of fast and slow reactions and to related that to the rate-limiting and near-equilibrium reactions. And finally I wanted to analyse the system in terms of feedback regulation, control characteristics and the general dynamics of the system. The latter concepts come primarily from electronics and were already being recognized in metabolic systems in the late 1950s. The discovery that hexokinase is allosterically regulated by its product (glucose 6-phosphate) gives one cause to wonder whether that is a master reaction since the conditions for a definite master reaction are violated; similarly for phosphofructokinase. Moreover, gluconeogenesis from lactate begins with the reversible reaction of lactate dehydrogenase; and there are many other pathways which begin with reversible reactions.

I presented my results at a colloquium on metabolic control held at the Johnson Foundation on May 20, 1965. It was a gathering of many great experimentalists and thinkers in the field of metabolic regulation. I believe I was the only pure theoretician there. In some respects, I felt like I was bringing coals to Newcastle, for Krebs could hardly be surprised to find that his intuitive thoughts regarding near-equilibrium reactions could be proved theoretically. And although I tried to present concepts rather than mathematics in my paper, I am sure many of the experimentalists could not wait for my talk to end. From their perspective I was saying nothing. What I did was to present them a method for determining the "rate-limiting" reaction and I told them that any reaction in a sequence might be rate controlling but that a sequence could also have all reactions with equal control. But nothing I said need have altered their own perceptions of how particular metabolic pathways were regulated *per se*.

I believe I had solved most of the problems I presented myself. And in retrospect, I think I have given a basic solution to the quandaries of past biomathematicians as regards a definition of fast and slow reactions and their relation to the master-reaction. In defining the control strength of a reaction by $^{d\ln v}/_{d\ln e}$, where e is the enzyme concentration I felt I had provided a useful method which experimentalists might apply to cellular extracts or that theoreticians could use, in analysing metabolic theory.

Many of my thoughts were related to electronic theory, especially the role of feedback theory and electronic control theory. And some of these thoughts had already invaded the thoughts of a few experimentalists. Thus, at that metabolic control symposium (1965), Hess notes that he has evidence showing that fructose 1,6-bisphosphate is a "feed-forward" activator of pyruvate kinase. In the same discussion, Bücker notes that he has evidence that there are two Master-reactions in muscle glycolysis, one at phosphofructokinase and the other at pyruvate kinase. I note these remarks because they show both a growing knowledge of feedback (or feed-forward) principles of electronics in the field of biochemistry, and as well even the concept that there could be two master-reactions in the same sequence.

Although I thought my theory was sound, I had no great vision that the experimentalists

would apply it the next day. In fact, being at the JF had given me the opportunity to suggest experiments to some of my colleagues; the general reaction was usually a chant: "Chain Higgins to the (lab) bench". I think that many experimentalists are now thinking the same thing, like "Chain all these theoreticians to the bench".

How did I Envisage the Future of Control Theory?

First of all, I did not anticipate any immediate future for control theory as I presented it at that time (1965). I was fully aware of the experimental difficulties in its application even to cellular extracts, let alone *in vivo*. The only realistic approach at present seems to be that of Barbara Wright (see Chapter 27 of this book), which in principle establishes a computer model of the *in vivo* system using control theory to analyse that system. But even that method is fraught with many pitfalls.

From the theoretical viewpoint, I had no intention of extending the theory to any purely theoretical model system. Perhaps I had heard too much of "Chain Higgins to the bench". As it happened, I was an associate editor of the *European Journal of Biochemistry* in the 1970s and I received the paper of Heinrich & Rapoport (1974) for review. First I kicked myself for having missed these very obvious summation theorems. But after some mental reflections, I was very pleased that someone, or anyone, had picked up on my concepts and thoughts.

Now, for the first time in my life, I am witness to a symposium where the number of theoreticians outweigh the number of experimentalists; where all those theoreticians are focussed on the same problem, and where even the experimentalists have their own old and new thoughts on metabolic control. To the theoreticians I should say that we have a long way to go in establishing a viable theory that experimentalists can easily apply. But I am also convinced that the theoretical foundations of current control theory is well founded, even if not the end-all.

From an electronics viewpoint the current control theory would be considered "small signal" analysis. Much work needs to be done to develop a theory for large signal changes as occur in glycolysis. Certainly, my original paper (Higgins, 1965) was concerned with control in the large as well as control in the small and with the role of feedback (or feed-forward) in determining the regulatory properties of a system. And in later papers, I analysed the properties of oscillatory reactions, which cannot be purely treated by small signal analysis because they represent limit cycles (Higgins, 1967) and the existence of multiple stationary states (Higgins, 1966) in rather simple feedback systems wherein the transfer between stationary states requires large signal analysis.

Let me summarize by saying that I believe this symposium to be an important milestone in the link between experimental and theoretical metabolic control. I believe that we have made some significant inroads into bringing the two together, but we are hardly there yet. To me, one of the most important aspects of this meeting is the gathering together of so many theoreticians, all focussed on the same problem. That is a rare thing in biochemistry and I doubt that it has ever been duplicated in the past. I can only hope that it continues.

Acknowledgements: In the preparation of this paper I am deeply indebted to Eva Christensen and Ruth Keris, both of whom worked overtime in order to help me bring this paper to completion. Their help is deeply appreciated.

REFERENCES

Burton, A. C. (1937) *J. Cell. Comp. Physiol. 9,* 1
Burton, A. C. (1939) *J. Cell Comp. Physiol. 14,* 327
Chance, B. (1943) *J. Biol. Chem. 151,* 553
Chance, B. (1949) *Waveforms*, MIT Radiation Laboratory Series, McGraw-Hill, New York
Chance, B., Williams, F. C., Yang, C. C., Busser, J. & Higgins, J. (1951) *Rev. Sci. Instr. 22,* 683
Chance, B. & Higgins, J. (1952) *Arch. Biochem. Biophys. 41,* 432
Chance, B., Williams, G. R., Holmes, W.F. & Higgins, J. (1955) *J. Biol. Chem. 217,* 439
Chance, B., Holmes, W., Higgins, J. & Connelly, C. M. (1958) *Nature 182,* 1190
Chance, B., Garfinkel, D., Higgins, J. & Hess, B. (1960) *J. Biol. Chem. 235,* 242
Chance, B., Estabrook, R. W. & Ghosh, A. (1964) *Proc. Natl. Acad. Sci. USA 51,* 124
Christiansen, J. H. (1935) *Z. Physik. Chem. 288,* 303
Crozier, W. J. (1924) *J. Gen. Physiol. 7,* 123
Garfinkel, D., Rutledge, J. D. & Higgins, J. (1961) *Comm. ACM. 4*, 559
Hammett, L. P. (1937) *Physical Organic Chemistry*, McGraw-Hill, New York
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89
Higgins, J. (1959) PhD Thesis, University of Pennsylvania
Higgins, J. (1961) in *Technique of Organic Chemistry*, Vol. VIII, Interscience, New York
Higgins, J. (1963) *Ann. New York Acad. Sci. 108,* 305
Higgins, J. (1964) *Proc. Natl. Acad. Sci. USA 51,* 989
Higgins, J. (1965) in *Control of Energy Metabolism* (Chance, B., Estabrook, R. W. & Williamson, J. R., eds.) Academic Press, New York
Higgins, J. (1966) in *Académie Royale de Médecine de Belgique*, Tome VI, Série in-8, 235
Higgins, J. (1967) *Ind. Eng. Chem. 59,* 19

Chapter 3

Control Analysis: a Theory that Works

JOHN W. PORTEOUS

METABOLIC MAPS of the early 1950s showed enzymes interacting only with their immediate substrates, products and coenzymes. How a cell *resisted* marked changes in its flux rates in response to changes in concentrations of external substrates (even when none of its enzymes was saturated), or *responded* to changes in the concentrations of external effectors (e.g. hormones) was a mystery.

It is not then surprising that part of the mystery was assumed to be solved by the discovery in 1956 of feedback inhibition of the activity of an early enzyme in a metabolic pathway by a late metabolite in the pathway, the subsequent isolation of numerous enzymes exhibiting cooperative and allosteric responses, and the publication of several mechanisms (see Cornish-Bowden, 1979) to account for these responses. The further discovery of hormone-initiated covalent modification of enzyme activity, and of interconvertible enzyme cascades, encouraged the view that metabolic control was beginning to be understood. The discovery of the *lac* operon in *Escherichia coli* (the prototype of many such mechanisms) showed that mechanisms existed for modifying the concentration rather than the activity of some enzymes; and again encouraged the view that metabolic regulation occurred via special mechanisms. Molecular biology has provided its own array of alleged control mechanisms. Upstream promoters, repressor proteins and DNA methylases (for example) are each said to control gene expression; and controversies about the relative merits of claims for control of polypeptide synthesis at the transcriptional and translational levels continue unabated. Molecular biologists rarely link these proximal events in gene expression to their effect on the phenotype of the intact metabolizing system; their views on metabolic control thus become divorced from those of geneticists (their closest academic allies), physiologists and a growing number of biochemists.

Aside from these many mechanisms, which are now widely assumed to be uniquely important in metabolic control, other distinct notions are simultaneously favoured. "Cross-over" of the concentrations of metabolites is still said to indicate a site of control even though the idea, as an unambiguous criterion, has been discredited (Heinrich & Rapoport,

John W. Porteous ● Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, UK

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

1974*b;* Heinrich *et al.,* 1977). It is also still held that only at steps "far from equilibrium" could control occur, conversely that control cannot occur at steps close to equilibrium. It is noticeable that "far" and "close" are never defined. This notion is based on false reasoning. Some authors discard all the above mechanisms and notions; they maintain that the concentration ratios of certain coenzymes (e.g. NAD^+/NADH) control metabolism. Such metabolite concentration ratios are free variables in a metabolic system. This notion is therefore false; one variable cannot be said to control another variable — they are both dependent on the parameters of the system.

What is Wrong with these Traditional Views of Metabolic Control?

First, no consensus identification of the nature or location of metabolic control has emerged from three decades of research along traditional lines. Second, there is nothing in the traditional treatments which will predict, even very approximately, the magnitude of any one system response in the intact metabolic system when any of the alleged mechanism operates; in other words, there is no means of ranking the relative importance of the various alleged controlling mechanisms when two or more operate simultaneously. Third, any relationship between control of flux rates and metabolite concentrations is absent. Fourth, no formal distinction is drawn between events within a system which play a role in metabolic control and events in the environment which might affect intracellular metabolic control. Fifth, insufficient account is taken of possible pleiotropic effects of any one alleged controlling mechanism.

It seems reasonable to reassess the usefulness of traditional views on metabolic control. A dispassionate view of the multitude of claims is that they share two faults:

1. Each claim about the site of metabolic control is related to just one small niche of a metabolic system; in the extreme, the niche contains just one enzyme with kinetic properties necessarily elucidated in the enzymologist's cuvette. The interactions of the niche components, via intermediate metabolites, with all the catalysts of the rest of the system are not taken into account.

2. Each claim is stated in terms that fail to relate the magnitude of the response of a *system* variable (e.g. a flux rate or the concentration of an internal metabolite) to a change in the magnitude of a parameter of the system (e.g. an enzyme activity or concentration) or to a change in magnitude of an external (environmental) parameter.

These two criticisms do not mean that we should cease to work on isolated components of a system, still less that we should ignore the findings of enzymologists. On the contrary, the enzymologist's observations are invaluable; they alert us to the range of responses which an enzyme could make (under defined conditions) to small finite changes in concentration of anyone of several molecules and ions with which it interacts so as to modify its activity. Without that information it would be difficult to make progress. The danger lies in using that

information *alone* in attempting to understand metabolic control. For an excellent example of the wide range of responses which can occur under different constraining conditions (and the possible danger of discarding observations which do not fit current ideas on control), see Cárdenas & Cornish-Bowden (1989; also Chapter 14 of this book).

Developing the Theory of Control Analysis

Control analysis is concerned with metabolic systems as we currently understand them; it recognizes (by studying metabolic maps) that every enzyme is flanked by and interacts with at least one substrate and at least one product in all metabolic systems, no matter how complex they may be. Because enzyme and carrier activities depend on the same kind of initial concentration-dependent non-covalent interaction between the catalyst and its substrates, they are treated alike; for simplicity of presentation, enzymes alone are mentioned in the following text. For typographical convenience only, reaction steps are taken to involve only single substrates and single products; the algebraic statements would be more complex, but keep the same form, if multiple substrates and products were allowed. Changes in enzyme concentrations and changes in enzyme specific activities are allowed for, no matter how they may be brought about (Kacser & Burns, 1973; Heinrich & Rapoport, 1974*ab*, 1975; Heinrich *et al.*, 1977); so too are non-catalysed diffusions of solutes (Kacser, 1983; also Heinrich in Chapter 28 of this book) within a cell compartment or across a membrane.

Control analysis shows that control of fluxes and metabolite concentrations is a systemic function (and not an inherent property of individual enzymes) and is necessarily distributed among all catalysts of the system; it is designed to enable the experimentalist to *measure* this distribution of control among the catalysts of the system under defined environmental conditions. If any of the traditional notions about metabolic control turned out to be true, control analysis would confirm this by measurement rather than by teleological argument or intuition. To enhance understanding of the operation of a metabolic system, events at each local catalysed step in a system are related to the global or systemic functions of the system. Explicit account is taken of the effect of changes in concentration of components of the environment surrounding the system on the control of any specified function of the system. The treatment is restricted to stationary or expanding steady-state systems (i.e. to those systems with which the majority of experimentalists are currently concerned). Treatment of oscillations is excluded; so too (in the present account) is exploration of the transients that connect the movement of a system from one steady state to another (but see Acerenza *et al.*, 1989, as well as Chapters 23-26 of this book).

The Specific Assumptions of Control Analysis

Only four assumptions are made in this introductory account:

1. The system can attain and, for the duration of an experiment, does maintain a stationary

or expanding steady state. (This condition can be met by almost all systems currently investigated).

2. Each catalysed step (a chemical transformation by an enzyme or a solute translocation by a carrier) may show a linear or nonlinear response of its rate to successive, small, finite changes in the concentration of any one of the participating metabolites; each step (catalysed or not) is taken to be potentially reversible and made display any form of kinetics. Equations describing the kinetics of each step, or sets of steps, are, however, not required and are not used; the theoretical constructs of control analysis, as described here, depend on our knowledge of the metabolic structure of biological systems and not on any mathematical descriptions of the kinetics of individual steps or sets of steps.

3. Each catalysed step is of first order in catalyst concentration, i.e. there is an *additive* relationship between enzyme concentration and enzyme activity. There is then an exact equivalence between catalyst concentration and catalyst activity. (See the note following the next paragraph). Some critics of control analysis seem to think that it deals only with changes in enzyme concentration: this assumption, however, makes it possible to treat changes in enzyme concentration and changes in enzyme activity in equivalent terms, no matter how either change is brought about.

4. Each catalysed step shows *independence* from all other catalysed steps, i.e. there are no *direct* interactions between any one enzyme and another (or others) that could affect the kinetics of any one member of such a complex of enzymes. [These simplifying assumptions (3-4) have now been eliminated, as described by Kacser *et al.* in Chapter 20 of this book; cf. Heinrich *et al.* (1977), and Giersch and co-workers in Chapter 30) but are retained for the present account].

Model, Theory and Experiment

Enzymology constructs a model, typically showing a non-covalent interaction between enzyme and substrates, followed by catalytic formation of products and release of free enzyme. On this basis, equations are derived that are intended to mimic the experimentally observed kinetics of the enzyme-catalysed reaction; if theory (the model with its equation) and experiment are in close accord, the model and equation are taken (at least as a working hypothesis) to be sound.

Control analysis also proceeds from a model, via "thought experiments", to experimental testing. The model for control analysis (e.g. Schemes 1 and 2) is based on examination of metabolic maps; it asserts that reactions occur at each step in the map and that each reaction is coupled to the preceding and succeeding steps by the intermediate metabolites that are common to each pair of steps. The theory is developed from two *different* observations: first, the enzymologist's *ad hoc* observation that, for an individual reaction step, the catalysed steady-state reaction rate (the dependent variable) responds (usually nonlinearly)

to successive small finite changes in concentration of any one metabolite of this step (a parameter of the enzymologist's reaction mixture) when each other component of the reaction mixture is held at a constant concentration (the parameter that is changed may be the concentration of a substrate, product, coenzyme, effector, etc of the individual reaction step). Changing the enzyme concentration (also a parameter in the enzymologist's reaction mixture) often results in a proportional change in the catalysed rate. Second, the geneticist's and cell biologist's *ad hoc* observation (Kacser & Burns, 1981) that successive small finite changes in any one enzyme concentration (a parameter in a system of enzymes) results in a nonlinear response in a system variable (e.g. a metabolic flux or a metabolite concentration in the system). The first of these two observations is thus concerned with individual steps in a metabolic system, the second with the behaviour of the whole system.

Control analysis therefore defines two different indices of responsiveness. The first (the *elasticity coefficient*), is a measure of the responsiveness of an individual step (a *local* reaction rate) to a small finite change in any one of the participating metabolites. The second (the *control coefficient*), is a measure of the responsiveness of a system function (e.g. a metabolite concentration within the system or a flux through part or all of the system) to small finite changes in a system parameter (an enzyme concentration or activity).

Definitions

Elasticity Coefficients. Fig. 1 shows typical plots of the response of reaction rate v to discrete changes in the concentration of one substrate S_a or S_b. Enzymologists derive equations to mimic such plots and define the plots in terms such as v, S_a, S_b, V (limiting rate) and K_m (Michaelis constant). This is legitimate but not obligatory. We can instead extract the necessary information (for measuring responsiveness) by drawing the tangential slope on any such plot at any point corresponding to the current value of S_a or S_b; the absolute value of the slope will depend on the units chosen for the measurements. Units of measurements are eliminated by scaling the slope by the factor S/v (at the current values of S and v) to yield a dimensionless elasticity coefficient (ε) defined (for the rate v_j of the jth reaction and its substrate concentration S_i) as follows:

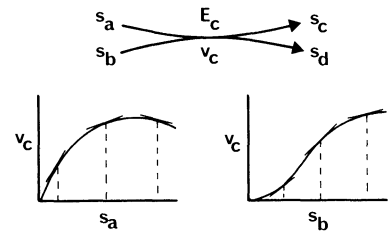


Figure 1. Typical plots are shown of the reaction rate v against the initial concentration S_a or S_b of one substrate in an enzymologist's reaction, all other components of the reaction mixture being kept constant in each case. Each plot represents the *response* of reaction rate (the dependent variable) to a change in one parameter (in these examples a single substrate concentration). The tangential slope at any given value of S_a or S_b is a measure of the responsiveness of v to small changes in S_a or S_b. Similar plots can be constructed showing the response of the forward reaction rate v to changes of the concentration of each of the products of the reaction: for normal kinetics, such plots would show negative slopes with respect to increases in product concentration.

$$\varepsilon_{S_i}^{v_j} = \frac{\partial v_j S_i}{\partial S_i v_j} = \frac{\partial v_j / v_j}{\partial S_i / S_i} = \frac{\partial \ln v_j}{\partial \ln S_i} \tag{1}$$

The first mode of expressing $\varepsilon_{S_i}^{v_j}$ in eqn. (1) shows the tangential slope scaled by S_i/v_j; thus, if the complete rate equation for a given step is known, all the elasticity coefficients at this step can be obtained by scaling the appropriate partial differentials of the rate equation. The second mode expresses the elasticity coefficient as the fractional change in the variable divided by the fractional change in the perturbing parameter; the third mode is the equivalent logarithmic form.

It is convenient to abbreviate the formal notation $(\varepsilon_{S_i}^{v_j})$ for a metabolite elasticity coefficient to ε_i^j . Note that definitions are necessarily stated as partial derivatives with infinitesimal changes denoted by ∂. In experimental practice small finite changes, denoted by δ, would be used. Given the value of ε_i^j, one may calculate from eqn. (1) the fractional response of the individual reaction rate v_j to a known fractional change in the concentration S_i. For any one reaction step v_j there will be as many elasticity coefficients as there are metabolites (substrates, products, coenzymes, effectors, etc) which are involved in that reaction. The value of any ε may be positive or negative, greater than or less than 1 (Fig. 1). It may be determined experimentally in the same way that values of V or K_m are determined but, for use in intact metabolic systems, such determinations must be carried out under conditions to which the enzyme would be exposed *in vivo*. The notation for the elasticity coefficient for enzyme E_i on its own reaction is $\varepsilon_{E_i}^j$ [= 1 by assumption 3 above].

Control Coefficients. A plot of the response of the flux, through a *system* of enzymes, to successive finite changes in the concentration of *any one* enzyme is non-linear (Kacser & Burns, 1981). A flux control coefficient C is therefore defined in the same mathematical form as an elasticity coefficient but obviously has a completely different role to play in understanding system behaviour; it is concerned with a systemic function, not a local reaction rate:

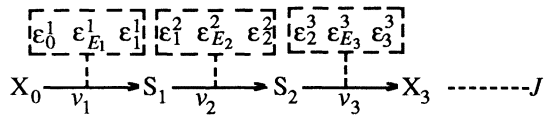
$$C_{E_i}^{J_k} = \frac{\partial J_k E_i}{\partial E_i J_k} = \frac{\partial J_k / J_k}{\partial E_i / E_i} = \frac{\partial \ln J_k}{\partial \ln E_i} \tag{2}$$

So long as it clear from the context that a *flux* control coefficient is meant, $C_{E_i}^{J_k}$ can be abbreviated to C_i^k. Given the value of C_i^k, it is then possible to calculate the fractional response of the particular flux J_k to a fractional change in the concentration or activity of the particular named enzyme E_i. For any one flux J_k there will as many flux control coefficients as there are enzymes and carriers in the whole metabolic system. The value of any one flux control coefficient may be positive or negative, greater or smaller than 1.0. The value of any such coefficient may be determined by direct experiment on the intact system, i.e. by varying one particular enzyme concentration or activity by small fractional increments or decrements (using any means available) and measuring the consequential fractional change in the chosen flux. Examples of this direct and of alternative indirect determinations of flux control coefficients will be found in publications quoted later in this chapter.

Metabolite control coefficients $C_{E_i}^S$ are defined in analogous fashion. For any one metabolite concentration S there will be as many such coefficients are there are enzymes and carriers in the system.

A Model Metabolic System

Metabolic control analysis proceeds stepwise from simple to more complex situations, considering first unbranched pathways, then branched pathways then conserved-moiety cyclic pathways. The very simplest unbranched pathway (Scheme 1) is modelled initially. Three elasticity coefficients are associated (as indicated) with *each* of the three catalysed steps of the model system. This minimal system comprises an external initial substrate X_0 and a final external product X_3; their concentrations X_0 and X_3 are kept constant despite the flux J through the pathway (this is commonly achieved in experimental practice in a number of well-known ways). These concentrations are then external parameters. The enzyme concentrations E_1, E_2, E_3 are internal parameters of the system; they could be varied (in concentration or activity) by direct intervention of the experimenter; in contrast, S_1 and S_2, the concentrations of the internal metabolites, are dependent variables — they cannot be altered other than by altering an external or internal parameter. Note that X_0 and X_3 are necessarily kept constant if we wish to discover and measure the response of the system to a change in any one of its internal parameters.



Scheme 1

The next four sections are concerned with the effects of changes occurring within such a system and within larger, more complex systems related to it.

Are Flux Control Coefficients Related to Elasticity Coefficients?

The three flux control coefficients of Scheme 1 are given by the following equations:

$$C_{E_1}^J = \frac{\varepsilon_1^2\varepsilon_2^3}{\varepsilon_1^2\varepsilon_2^3 - \varepsilon_1^1\varepsilon_2^3 + \varepsilon_1^1\varepsilon_2^2} \tag{3}$$

$$C_{E_2}^J = \frac{-\varepsilon_1^1\varepsilon_2^3}{\varepsilon_1^2\varepsilon_2^3 - \varepsilon_1^1\varepsilon_2^3 + \varepsilon_1^1\varepsilon_2^2} \tag{4}$$

$$C_{E_3}^J = \frac{\varepsilon_1^1\varepsilon_2^2}{\varepsilon_1^2\varepsilon_2^3 - \varepsilon_1^1\varepsilon_2^3 + \varepsilon_1^1\varepsilon_2^2} \tag{5}$$

Derivation of these equations employs the same kind of arguments used by Kacser & Burns (1973: Appendix B), Kacser (1983: divided pathways), Fell & Sauro (1985), and Hofmeyr *et al.* (1986; Appendix C).

One must avoid being misled by the presence of minus signs in these equations: in ordinary circumstances (when considering concentration ranges where substrate inhibition and product activation are not evident) *all of the products of elasticities, and hence all of the flux control control coefficients, are positive.* This is because each product elasticity coefficient (ε_1^1 and ε_2^2) is negative whereas all other (substrate) elasticity coefficients are positive under these circumstances.

Because X_0 and X_3 were *defined* as external parameters that are held constant, it follows that $\delta X_0/X_0 = \delta X_3/X_3 = 0$ by definition, and in consequence ε_0^1 and ε_3^3 do not appear in any of eqns.(3-5); furthermore, because all $\varepsilon_{E_i}^i = 1$ (Assumption 3, above), the enzyme elasticities do not appear explicitly in these equations. All the other elasticity coefficients of Scheme 1 appear in each denominator term. Furthermore, the three denominators are identical, whereas each numerator is unique to a particular flux control coefficient and is one of the denominator terms. It follows that each flux control coefficient will be different in magnitude (i.e. each enzyme of the system will exert a different, quantifiable, degree of control over the flux).

It is immediately obvious that the flux control coefficient (a measure of the control exerted by any one enzyme on a given flux) is not a unique property of that enzyme; it is a systemic property determined by the responsiveness of each catalysed step (measured by the magnitude of the associated elasticity coefficients) to any small, finite, changes in the concentrations of the molecules which participate in that step (included is any change in the concentration, or the equivalent change in activity, of the enzyme). The magnitude of each metabolite elasticity coefficient varies if the metabolite concentrations change substantially (Fig. 1). It follows that the magnitudes of individual flux control coefficients [eqns. (3-5)] may also change when the same metabolic system is examined under markedly different experimental conditions. There are, however, constraints upon such changes in the flux (and metabolite) control coefficients.

The Summation and Connectivity Properties

The sum of three numerator terms of eqns. (3-5) equals the common denominator; that is, the sum of the three flux control coefficients is 1.0. If Scheme 1 were expanded by inserting one more enzyme and one more internal metabolite, there would then be four flux control coefficients; the denominators of the right hand side of four equations would now each contain four terms (each containing three elasticities) but each unique numerator term would still be one of the four denominator terms. The sum of the four flux control coefficients would again be 1.0. This flux control summation property applies to all metabolic systems, no matter how complex they may be; it follows that if, as conditions change, one or more flux control coefficients increase in magnitude, another or others must decrease so as to satisfy the summation property which is expressed as follows:

$$\sum_{i=1}^{n} C_{E_i}^{J} = 1$$

Eqn. (2) above shows that a large value for a flux control coefficient would be expected when the fractional flux response is large for a small fractional change in a given enzyme concentration or activity. The challenge to the experimenter is to discover which enzymes in a given system possess large and which possess small flux control coefficients, so discovering which enzymes are more and which less important in controlling that particular flux under defined experimental conditions.

The corresponding summation property of the metabolic control coefficients, which likewise applies a metabolic system of any complexity (Heinrich & Rapoport, 1975; Heinrich, Rapoport & Rapoport, 1977), is expressed as follows:

$$\sum_{i=1}^{n} C_{E_i}^{S_j} = 0$$

If eqn. (3) is divided by eqn. (4), and eqn. (4) in turn is divided by eqn. (5) the following ratios are found:

$$C_{E_1}^{J}/C_{E_2}^{J} = \varepsilon_1^2/(-\varepsilon_1^1) \quad \text{and} \quad C_{E_2}^{J}/C_{E_3}^{J} = \varepsilon_2^3/(-\varepsilon_2^2)$$

That is, the ratio of the flux control coefficients for two adjacent enzymes is inversely proportional to the ratio of the elasticity coefficients for the two enzymes with respect to their common intermediate metabolite (see Scheme 1 above). (As with the earlier equations one should not be misled by the minus signs: both ε_1^1 and ε_2^2 are normally negative, so both quantities in parentheses are positive, and all of the ratios in these equations are positive). The general statement of this relationship is called the *flux control connectivity property:*

$$\sum_{i=1}^{n} C_{E_i}^{J} \varepsilon_s^i = 0$$

The elasticity coefficients in this summation include those for any allosteric effectors. The general formulation allows also for the instance where one metabolite interacts with more than two enzymes (e.g. at a branch point in a metabolic pathway or in a feedback loop). The flux control connectivity property of a metabolic system explains how it comes about that the magnitudes of the flux control coefficients will redistribute if any one of them changes. The corresponding general formulation for the *metabolite control connectivity property* (Westerhoff & Chen, 1984) is as follows:

$$\sum_{i=1}^{n} C_{E_i}^{S_m} \varepsilon_s^i = -\delta_{mk}$$

where δ_{mk} is the Kronecker symbol and equals 1 if $m = k$ and 0 if $m \neq k$; m and k refer to any of the independently variable internal metabolites of the system. The summation is (as before) over all n enzymes of the system. See Giersch (1988*ab*) for further discussion

of the summation and connectivity properties. In Chapter 19 of this book Hofmeyr describes a systematic diagrammatic representation of the connectivities within any metabolic structure.

Is There a General Relationship Between Flux and Metabolite Control Coefficients?

Heinrich & Rapoport (1974*a*, 1975) demonstrated a complex but precise relationship between flux control coefficients and metabolite control coefficients. It will not be reproduced here but it is important in again emphasizing the co-ordinated interactions within a metabolic system; the relationship also has practical applications (Rapoport *et al.*, 1974; Rapoport *et al.*, 1976, Wanders *et al.*, 1984).

Extension of Control Analysis to Branched Pathways, Loops and Moiety-conserved Cycles

There is not space here to deal with these extensions. The original papers on branched pathways (Kacser, 1983; Fell & Sauro, 1985, Sauro, Small & Fell, 1987; Westerhoff & Kell, 1987; Giersch 1988*ac;* Small & Fell, 1989) should be consulted. The essential points are as follows:

1. The simplest branched pathway consists of three unbranched segments joined at a metabolite which interacts with three rather than two adjacent enzymes. A model system might therefore show an input flux leading to the formation of the "branch point" metabolite and two output fluxes which consume this metabolite; any one of these three fluxes may be defined as the reference flux.

2. Sets of equations like eqns. (3-5) above (one set in respect of each reference flux) can still be written but some of the elasticity coefficients must now be scaled by a factor α or $1 - \alpha$, where α is the proportion of an input flux that appears in one of two output fluxes beyond the branch point.

3. The partitioning ratio of the two output fluxes $\alpha/(1 - \alpha)$ is a systemic characteristic [Fell & Sauro, 1985; their eqn. (A4)] - not a function of the "branch-point" enzymes alone. (This observation contradicts a widespread but unproved contention in traditional views on metabolic control: see Atkinson in Chapter 36 of this book).

4. Negative control coefficients are generated when branching of pathways occurs.

5. The summation and connectivity properties still apply.

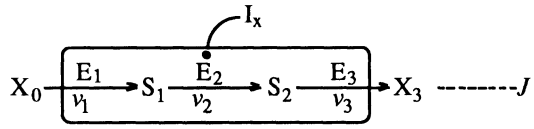
Similarly, the original papers on metabolic loops and conserved-moiety cycles should be consulted (Fell & Sauro, 1985; Sorribas & Bartrons, 1986; Hofmeyr *et al.*, 1986; Small & Fell, 1987; Small & Fell, 1989). Again, the summation and connectivity properties still apply but some additional properties of metabolic loops and conserved-moiety cycles emerge [particular care is required in distinguishing between non-conserved-moiety cycles and conserved-moiety cycles. Derr (1986) refers to the Krebs cycle as a typical example of a

moiety-conserved cycle; this is unlikely ever to be true because citrate, α-oxoglutarate, succinate and oxaloacetate may enter, and exit from, the cycle independently of each other and conceivably at rates not very different from the cycling rate (see Hofmeyr *et al.*, 1986)].

Fell and colleagues have devised matrix algebraic methods that greatly simplify the calculation of flux and metabolite control coefficients from elasticity coefficients (see Chapter 9 of this book). The utility of these matrix solutions for control coefficients depends, of course, on the availability of reliable estimates of all the relevant elasticity coefficients.

External, Independently Variable (Parametric) Effectors

The whole of the treatment thus far has been concerned with defining an experimentally accessible index of the relative importance of individual enzymes (the control coefficients) in controlling a systemic function such as a flux or a metabolite concentration; and with demonstrating the connection between the responsiveness of local events (measured by the magnitude of elasticity coefficients) and the responsiveness of the whole system (measured by the magnitude of the control coefficients). Scheme 1 served as the elementary model. During the "thought experiments" described in the preceding sections, nothing was said or implied about the means by which the concentration or activity of any one enzyme was modulated; the treatment was thus entirely general. We can therefore next ask how the system would respond to modulation of any one enzyme activity by an external agent. Scheme 2 illustrates the same metabolic Scheme 1 (without showing the internal elasticity coefficients) but with the addition of an external effector, e.g. an inhibitor I_x. The concentration of I_x is set or changed by the experimenter, or by the activity of an effectively independent system; it is not affected by the activity of an effectively independent system and is therefore a parameter (Kacser & Burns, 1973) or an independent variable.



Scheme 2

It is now necessary to define a special kind of elasticity coefficient [a *kappa elasticity coefficient,* originally called the *controllability coefficient* and symbolized κ (Kacser & Burns, 1973)]:

$$\kappa\varepsilon_{I_x}^{v_2} = \frac{\partial v_2/v_2}{\partial I_x/I_x} \qquad (6)$$

The kappa elasticity coefficient is defined in the same manner as other elasticity coefficients but has a special role to play; it provides a measure of responsiveness of a named internal reaction rate (v_2) to a small finite change in the concentration of a specified external or *independently variable* effector (I_x). Kappa elasticities do *not* contribute to the magnitude of flux or metabolite control coefficients; they do *not* appear in equations such as eqns. (3-5) above.

It is also necessary to define a special kind of system coefficient called a *response coefficient R*, which does *not* contribute to the summation property and does *not* participate in the connectivity property:

$$R^J_{I_x} = \frac{\partial J/J}{\partial I_x/I_x} \tag{7}$$

The response coefficient is defined in the same way as a control coefficient but again plays a special role; it provides a measure of the responsiveness of a flux (e.g. J in Scheme 2) within or through a system to small finite changes in an *external parameter* such as the concentration I_x of an effector; i.e. it measures the effect of a change in concentration of this effector on a system property (e.g. a flux) when the effector interacts with one particular enzyme in the system. The magnitude of any response coefficient can be shown to be the product of the current value of the flux control coefficient for the enzyme and the kappa elasticity coefficient for that enzyme with respect to the effector [Kacser & Burns, 1973; see Kacser & Porteous (1987) for a restatement using the current notation and terminology, that of Burns *et al.* (1985)]:

$$R^J_{I_x} = C^J_{E_2} \kappa\varepsilon^2_{I_x} \tag{8}$$

Substituting in eqn (7), it follows that a measured fractional change in the flux J would be the product of three factors:

$$\frac{\delta J}{J} = C^J_{E_2} \kappa\varepsilon^2_{I_x} \frac{\delta I_x}{I_x} \tag{9}$$

Because the concentration of the external substrate X_0 of Scheme 2 is a parameter (by definition), a fractional change in a particular flux J consequent on a small finite change in X_0 (with all other parameters kept constant) would also be the product of three factors:

$$\frac{\delta J}{J} = C^J_{E_1} \kappa\varepsilon^1_{I_x} \frac{\delta X_0}{X_0} \tag{10}$$

Eqns (6-10) apply irrespective of the nature of the effector or the complexity of the system involved; it is only necessary that the concentration of the effector (I_x or X_0 in this example) be independent of the activity of the system under investigation. Interaction by the external effector could be with any one of the enzymes or carriers within the system (including those or on within the cell surface membrane). See Groen *et al.* (1982), Hofmeyr *et al.* (1986) and Cárdenas & Cornish-Bowden (1989; also Chapter 14 in this book) for examples of the application of these equations.

Control Analysis in Experimental Practice

Table 1 lists techniques in common use for varying the activity or concentration of selected enzymes and carriers within intact metabolic systems. Table 2 lists groups of papers in

Table 1. Methods available for modulating the activity or concentration of selected enzymes in metabolic systems

	Method used	Resulting change[1]
1.	Change gene dose	Concentration of expressed enzyme
2.	Select mutant for "structural" gene; site-specific mutation	Activity of expressed enzyme
3.	Select mutant for "regulator" gene	Concentration(s) of expressed enzyme(s)
4.	Introduce plasmid carrying	Concentration(s) of expressed enzyme(s)
	(a) a structural gene or	(a) increased or
	(b) expressing anti-sense mRNA	(b) decreased
5.	Add single enzyme to cell-free enzyme system	Concentration of one enzyme
6.	Change concentration of external (parametric) effector[2]	Activity or concentration of (internal) enzyme
7.	Change concentration of external inducer, structural gene(s) expressed or not[2]	Concentrations of corresponding enzymes

[1] Changes in enzyme concentration are effectively changes in enzyme activity

[2] If method 6 or 7 is employed, eqns. (6-9) in the text necessarily apply. These equations may also be applicable elsewhere, provided the modulating effector is independent of the system and thus involves a kappa elasticity (Hofmeyr *et al.*, 1986; Cárdenas & Cornish-Bowden, 1989). Eqns. (6-9) do not therefore apply to the modulations employed in methods 1-5 of this Table.

Table 2. Publications in which Control Analysis has been used

System	References[1]
Glycolysis	
in erythrocytes	Rapoport *et al.*, 1974, 1976; Rapoport & Heinrich, 1975; Heinrich, 1985; CHAPTER 28
in yeast	Brindle, 1988
in liver extract	Torres *et al.*, 1986, 1988; CHAPTER 18
Gluconeogenesis in hepatocytes	Groen *et al.*, 1983, 1986; CHAPTER 6
Amino acid metabolism in hepatocytes	Salter *et al.*, 1986; CHAPTER 32
De novo purine biosynthesis in human T-cells	Smith *et al.* in CHAPTER 33
Respiration in hepatocyte and yeast mitochondria	Groen *et al.*, 1982; Tager *et al.*, 1983; Gellerich *et al.*, 1983; Wanders *et al.*, 1984; Holness *et al.*, 1984; Bohnensack, 1985; Westerhoff, 1985; Mazat *et al.*, 1986; Pryor *et al.*, 1987; Westerhoff *et al.*, 1987; Moreno-Sánchez *et al.*, 1988; Brand *et al.*, 1988
Citrulline synthesis in hepatocyte mitochondria	Wanders *et al*, 1984
Arginine synthesis in *Neurospora crassa*	Flint *et al.*, 1981
Photosynthetic CO_2 fixation	Woodrow, 1986; Kruckeberg *et al.*, 1989; Stitt *et al.* in CHAPTER 31
mRNA translation in reticulocytes	Heinrich & Rapoport, 1980
Bacterial growth	Dean *et al.*, 1986; CHAPTER 34; Westerhoff & van Dam, 1987
Expression of the genome	Westerhoff *et al.* in CHAPTER 35

[1]References to CHAPTERS are to this book

which control analysis has been applied. Examples from most groups are fully discussed elsewhere in this book. A few general comments are in place. Several examples are now available showing that:

1. A set of flux or metabolite control coefficients can be obtained for all or most of the enzymes (and any carriers) in common pathways;

2. The magnitudes of these coefficients change (subject to the constraint of the summation property) if experimental conditions vary substantially;

3. Some steps close to equilibrium exert measurable control on system activity and some steps far from equilibrium do not necessarily exert much control;

4. Genetic manipulations leading to substantial under-production or marked over-production of a single enzyme do not necessarily have any detectable effect on some fluxes to product formation, or even on cell growth;

5. When elasticity coefficients are also measured, it is possible to understand how it comes about that some steps possess large and others small control coefficients.

These last demonstrations are particularly satisfying because one of the aims of control analysis (Kacser & Burns, 1973; see also Kacser & Porteous, 1983) was to understand system functions in terms of events at all the local steps in the system.

These are remarkable achievements and entirely in line with the predictions of control analysis; they suggest that it is time to abandon the traditional descriptive approach to metabolic control and especially the dichotomies implied by such terms as regulatory and non-regulatory enzymes, reversible and irreversible reactions (usually but erroneously stated as reversible and irreversible enzymes). It is, of course, not reasonable to expect every detail of these pioneering results to remain unchallenged. For example, Groen *et al.* (1982) claimed that at low respiration rates the proton leak alone controlled respiration by non-phosphorylating mitochondria; it is now clear that control under these circumstances is shared between the proton leak and the respiratory chain (Brand *et al.*, 1988).

Conditions for the Use of Inhibitors in Control Analysis

In each of these successful applications of control analysis, the experimentalists have necessarily modulated a parameter of the system under investigation. One such modulation technique involves titrating a particular enzyme within the system with an inhibitor of proved characteristics. Any such approach requires particular attention to all three limitations of the method (Kacser & Burns, 1973): (1) The concentration of the inhibitor at the point of action must be known; if the external concentration is all that can be measured, then the internal concentration should be some known function of this internal concentration. (2) The inhibitor must be specific to one enzyme only. (3) Its inhibition kinetics must be proved for that enzyme.

In Chapter 29 of this book, Berry and colleagues argue that control analysis proved unsatisfactory in the analysis of their inhibition experiments. However, they provide no evidence that each of the inhibitors they employed was specific to a single enzyme in their system. If they were not specific, that control analysis failed in their hands would be readily explained: condition (2) would not be satisfied. There is, furthermore, no indication that the concentration of rotenone at the points of inhibition was known in their experiments. Nor is it clear that the inhibitors used by them were proved to be irreversible inhibitors of the individual enzymes particular to their experimental system; if they were not, even if one assumes that all other conditions for the use of inhibitors in control analysis were satisfied, the equation they used for obtaining the magnitude of the flux control coefficient from the inhibition data would be inappropriate (see Derr, 1986).

One further aspect of the use of inhibitors in control analysis has perhaps not been realized or sufficiently emphasized in the past. If the inhibition plot is sigmoid, and if the descending, non-linear, part of the plot reaches zero flux or approaches it closely, then it is legitimate (Groen *et al.*, 1982) to extrapolate this near-linear part of the plot onto the inhibitor concentration axis to obtain an estimate of I_{max}, the concentration of inhibitor required to inhibit the enzyme completely. If, on the contrary, an inverted hyperbolic plot is obtained (Berry and co-workers in Chapter 29 of this book, Fig. 1), the tangent to the plot at $I = 0$ will give an estimate of $\partial J/\partial I$. But to extrapolate this *same tangent* onto the inhibitor concentration axis will inevitably give a flux control coefficient of 1.0; this inevitability arises from the geometry of the plot and the tangent at $I = 0$, rather than from the inhibition of the enzyme and the consequential effects on a given system flux. This inevitability will arise (in any experimental system) for every enzyme that exhibits such an inverted hyperbolic plot if the formula $C_E^J = I_{max}(\partial J/\partial I)/J$ (Groen *et al.*, 1982; Derr, 1986) is applied and I_{max} estimated in this incorrect manner. Berry and colleagues are thus not justified in suggesting that their results reveal a paradox in control analysis. Their alternative conclusion "that the enzymes of the pathway must be highly channelled" seems insecure on the evidence available.

Analogous objections can be made to their conclusion from Fig. 2 of Chapter 29 that "the flux control coefficient for ATP synthase is 0". To obtain a reasonable estimate of the initial slope $\partial J/\partial I$ of these plots, many more plotting points would be required close to $I = 0$. It is more likely that the flux control coefficient for ATP synthase had a very small positive value. On present evidence from this figure it would be unwise to claim more than that this flux control coefficient approached zero. It is, in any case, inappropriate to conclude "that an excess of ATP synthase is present": this confuses the terminology and concepts of traditional views on metabolic control with those of control analysis (Kacser & Burns, 1973, 1981; Heinrich *et al.*, 1977).

Other Systematic Treatments of Metabolic Control

The approach to control analysis described here is due to Kacser and his collaborators. A mathematically more complex but more generalized approach to control analysis was used by the Berlin group (for a review see Heinrich *et al.*, 1977) and applied to several aspects of

human erythrocyte behaviour (see Chapter 28 by Heinrich in this book). Westerhoff (1985) and Westerhoff & van Dam (1987) have pioneered the translation of the kinetic approach to control analysis into an energetic approach. Savageau, starting in 1969, has published a long series of papers on *biochemical systems theory* (based on his power-law approximation) describing the behaviour of a range of model systems and comparing model behaviour with that of biological systems [see Savageau (1976), as well as Chapters 4 and 5 in this book]. Reder and Mazat (see Chapters 7 and 8 in this book) have described a *structural approach* to the behaviour of metabolic systems.

In Chapter 6 of this book Groen and Westerhoff make a systematic comparison between several of these methods, applying them in turn to the control of gluconeogenesis in rat hepatocytes, and showing that despite variations in emphasis, language and symbolism, they actually embody essentially the same mathematical ideas and lead to entirely compatible results. Which of them gives most insight into the operation of biological insight, which is most suited to computer modelling, and which is most readily applied to bench experiments should become clearer from the chapters that follow, particularly those in Section VI.

REFERENCES

Acerenza, L. Sauro, H. M. & Kacser, H. (1989) *J. Theor. Biol. 137,* 423-444
Bohnensack, R. (1985) *Biomed. Biochim. Acta 44,* 853-862
Brand, M. D., Hafner, R. P. & Brown, G. C. (1988) *Biochem. J. 255,* 535-539
Brindle, K. M. (1988) *Biochemistry 27,* 6187-6196
Cárdenas, M. L. & Cornish-Bowden, A. (1989) *Biochem. J. 257,* 339-345
Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics,* Butterworths, London & Boston
Dean, A. M., Dykhuizen, D. E. & Hartl, D. L. (1986) *Genet. Res. Camb. 48,* 1-8
Derr, R. F. (1986) *Biochem. Arch. 2,* 31-44
Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561
Flint, H. J., Tateson, R. W., Barthelmess, I. B., Porteous, D. J., Donachie, W. B. & Kacser, H. (1981) *Biochem. J. 200,* 231-246
Gellerich, F. N., Bohnensack, R. & Kunz, W. (1983) *Biochim. Biophys. Acta 722,* 381-391
Giersch, C. (1988*a*) *Eur. J. Biochem. 174,* 509-513
Giersch, C. (1988*b*) *Eur. J. Biochem. 174,* 515-519
Giersch, C. (1988*c*) *J. Theor. Biol. 134,* 451-462
Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. & Tager, J. M. (1982) *J. Biol. Chem. 257,* 2754-2757
Groen, A. K., Vervoorn, R. C., van der Meer, R. & Tager, J. M. (1983) *J. Biol. Chem. 258, 14346-14353*
Groen, A. K., van Roermund, C., Vervoorn, R. C. & Tager, J. M. (1982) *Biochem. J. 237,* 379-389
Heinrich, R. & Rapoport, T. A. (1974*a*) *Eur. J. Biochem. 42,* 89-95
Heinrich, R. & Rapoport, T. A. (1974*b*) *Eur. J. Biochem. 42,* 97-105
Heinrich, R. & Rapoport, T. A. (1975) *BioSystems 7,* 130-136
Heinrich, R., Rapoport, S. M. & Rapoport, T. A. (1977) *Prog. Biophys. Molec. Biol. 32,* 1-82
Heinrich, R. & Rapoport, T. A. (1980) *J. Theor. Biol. 86,* 279-313
Hofmeyr, J.-H. S., Kacser, H. & van der Merwe, K. J. (1986) *Eur. J. Biochem. 155,* 631-641
Holness, M., Crespo-Armas, A. & Mowbray, J. (1986) *FEBS Lett. 177,* 231-235
Kacser, H. (1983) *Biochem. Soc. Trans. 11,* 35-40
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Kacser, H. & Burns, J. A. (1981) *Genetics 97,* 639-666

Kacser, H. & Porteous, J. W. (1987) *Trends Biochem. Sci. 12,* 5-14
Kruckeberg, A. L., Neuhaus, H. E., Feil, R., Gottlieb, L. D. & Stitt, M. (1989) *Biochem. J. 261,* 457-467
Mazat, J.-P., Jean-Bart, E., Rigoulet, M. & Guerin, B. (1986) *Biochim. Biophys. Acta 849,* 7-15
Moreno-Sánchez, R., Espinosa-Garcia, M. T. & Raya, J. C. (1986) in *Integration of Mitochondrial Function* (Lemasters, J. J., Hackenbrock, C. R., Thurman, R. G. & Westerhoff, H. V., eds.) pp. 297-304, Plenum, New York
Pryor, H. J., Smyth, J. E., Quinlan, P. T. & Halestrap, A. P. (1987) *Biochem. J. 247,* 449-457
Rapoport, T. A., Heinrich, R., Jacobasch, G. & Rapoport, S. M. (1974) *Eur. J. Biochem. 42,* 107-120
Rapoport, T. A. & Heinrich, R. (1975) *BioSystems 7,* 120-129
Rapoport, T. A. & Heinrich, R. (1976) *Biochem. J. 154,* 449-469
Salter, M., Knowles, R. G. & Pogson, C. I. (1986) *Biochem. J. 234,* 635-647
Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221
Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts
Small, J. R. & Fell, D. A. (1989) *J. Theor. Biol. 136,* 181-197
Sorribas, A. & Bartrons, R. (1986) *Eur. J. Biochem. 158,* 107-115
Tager, J. M., Wanders, R. J. A., Groen, A. K., Kunz, W., Bohnensack, R., Kuster, U., Letko, G., Bohme, G., Duszynski, J. & Wojtczak, L. (1983) *FEBS Lett. 151,* 1-9
Torres, N. V., Mateo, F., Meléndez-Hevia, E. & Kacser, H. (1986) *Biochem. J. 234,* 169-174
Torres, N. V., Mateo, F. & Meléndez-Hevia, E. (1988) *FEBS Lett. 233,* 83-86
Wanders, R. J. A., van Roermund, C. W. T. & Meijer, A. J. (1984) *Eur. J. Biochem. 142,* 247-254
Westerhoff, H. V. (1985) *Biomed. Biochim. Acta 44,* 929-941
Westerhoff, H. V. & Chen, Y.-D. (1984) *Eur. J. Biochem. 142,* 425-430
Westerhoff, H. V. & Kell, D. B. (1987) *Biotechnol. Bioeng. 30,* 101-107
Westerhoff, H. V. & van Dam, K. (1987) *Thermodynamics and Control of Biological Free Energy Transduction,* Elsevier, Amsterdam
Westerhoff, H. V., Plomp, P. J. A. M., Groen, A. K., Wanders, R. J. A., Bode, J. A. & van Dam, K. (1987) *Arch. Biochem. Biophys. 257,* 154-169
Woodrow, I. E. (1986) *Biochim. Biophys. Acta 851,* 181-192

Chapter 4

Biochemical Systems Theory: Alternative Views of Metabolic Control

MICHAEL A. SAVAGEAU

AN APPROPRIATE language or formalism for the analysis of complex biochemical systems has been sought for several decades. The necessity for such a formalism results from the large number of interacting components in biochemical systems and the complex nonlinear character of these interactions. The Power-Law Formalism (Savageau, 1969*b*) is an example of such a language that underlies several recent attempts to develop an understanding of integrated biochemical systems. This formalism provides the basis for a theory, which is called *Biochemical Systems Theory*. Several different strategies of representation are possible within Biochemical Systems Theory. Among these, the "*S*-system" representation is the most useful, as judged by a variety of objective criteria (Sorribas & Savageau, 1989*abc*). This chapter first describes the predominant features of the *S*-system representation. The mathematical form of the *S*-system is deduced from the biochemical network in a straightforward fashion. The parameters of the *S*-system — rate constants and kinetic orders — are readily related to experimental data. The steady-state behaviour is characterized by a set of linear algebraic equations that can be solved symbolically or numerically. The differential equations that characterize the dynamic behaviour can be solved with very efficient numerical techniques. Methods for making well-controlled comparisons of alternative designs have been developed and applied to several classes of biochemical systems. In many cases these applications have led to more specialized theories with strong predictive capabilities. Specific predictions of these theories have been confirmed by experimental results from a number of independent laboratories. This chapter presents detailed comparisons between the *S*-system representation and other variants within Biochemical Systems Theory. These comparisons are made on the basis of objective criteria that characterize the efficiency, power, clarity and scope of each representation. Two of the variants within Biochemical Systems Theory are intimately related to other approaches for analysing biochemical systems, namely the *Metabolic Control Theory* of Kacser & Burns (1973) and of Heinrich & Rapoport (1974) and the *Flux-Oriented Theory* of Crabtree & Newsholme (1987). It is hoped that the comparisons presented here will result in a deeper understanding of the relationships between these variants.

Michael A. Savageau ● Department of Microbiology and Immunology, The University of Michigan, Ann Arbor, Michigan 48109-0620, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

Background

The explosive growth of molecular biology in the past 30 years is often referred to as "The Biological Revolution". As a result of this intensely focussed activity we are now in the position of knowing a great deal about the molecular constituents for a number of the simpler organisms — a number of viruses and the common bacterium *Escherichia coli* in particular. In the case of *E. coli* we now know on the order of 80 percent of all its genes and proteins. It is easy to perceive that in the next few years we will know all of the molecular elements of this organism. This is testimony to the power and efficiency of the reductionist approach. We have learned a tremendous amount in the past several decades, primarily because of this approach; it is clearly an essential tool. One need only look around to see how the world view of molecular biology permeates the biological disciplines, courses, and indeed the daily newspapers.

Any respectable reductionist is also a reconstructionist. By this I mean that if you ask a reductionist what his or her objective is, you will find that it is to reduce complex systems to their elemental units in order to characterize them, and once this is accomplished, to use this knowledge for reconstructing an understanding of the intact entity with which the investigation started. The problem is that the reconstructionist phase of this program is seldom carried out. This is due in part to preoccupation with the task of elucidating the molecular elements. There is still much to be done in this regard, and there are still great rewards for pursuing this task as long as the reductionist paradigm remains dominant in biology.

Paradoxically, it is at the very height of its success that the weaknesses of this paradigm are becoming increasingly apparent. It is now obvious that, as noted above, we shall soon have the complete parts catalogue for some organisms. Yet, by comparison, we still know relatively little about the integrated system, what makes it a living cell, or how it will respond to novel environments and to specific changes in its molecular constitution. In short, our knowledge is still fragmented and descriptive; we have almost no understanding of the "design principles" that govern biological systems. The reasons for this failure of reconstruction are more fundamental than preoccupation with other tasks. The reductionist paradigm itself is inherently unable to deal with these issues; a radically different and complementary "systems" approach is required. To see why this is so one need only look to the knowledge of the cell that has been revealed by advances in molecular biology.

Cellular components exhibit interactions that are associative rather than additive. These give rise to heterogeneous systems with rather complex nonlinearities, which account for most of the interesting properties of living cells. Associative interactions also produce rich hierarchical networks that are strongly coupled and highly organized. This structure must be taken into account if one is to understand the integrated behaviour of the cell. The network of interactions in a typical cell is characterized by thousands of variables and poses an enormous book-keeping problem that requires the aid of systematic methods that can only be provided by mathematics.

Table 1. Roots of the Power-Law Formalism

Kinetics	Biology	Networks
	1638 Galileo	1637 Descartes
		1687 Newton, Leibniz
		1736 Euler
		1750 Laplace
		1822 Fourier
	1838 Rameaux and Sarrus	
	1847 Bergmann	
		1858 Cayley
1867 Guldberg and Waage	1864 Spencer	1860 Kirchhoff
1902 Brown		
1903 Henri		
1913 Michaelis and Menten	1917 Thompson	
1923 Hill		
1925 Briggs and Haldane		
	1932 Huxley, Teissier	
		1945 Bode
		1948 Mason
1956 Umbarger	1950 Needham	1953 Hearon
1957 Dalziel		
1958 Alberty		
	1960 Bertalanffy	

Mathematical Formalisms

An appropriate language or formalism with which to analyse complex biochemical systems has been sought for several decades. The only formalisms known to be capable of dealing with the distinctive characteristics of complex biochemical systems are mathematical. Two well-known mathematical formalisms that frequently have been used for analysis of biochemical systems are the *Linear Formalism* and the *Michaelis-Menten Formalism.*

The Linear Formalism is among the best understood and developed mathematical structures. A linearized description of a biochemical system can be efficiently treated mathematically in many different ways, even when there are hundreds of system components. It is a general symbolic formalism guaranteed to be valid at least over some restricted range of the concentration variables. However, the variables in biochemical systems vary over ranges wide enough to produce highly nonlinear behaviour and therefore the Linear Formalism, which cannot represent known nonlinear properties of biochemical systems, is inappropriate.

The Michaelis-Menten Formalism, on the other hand, approximates many individual reactions reasonably well *in vitro*. Descriptions in this formalism are readily utilized as long as only one enzyme or a system of very few enzymes is being studied. However, under physiological conditions, each enzyme is not isolated, but interacts with other enzymes and structures embedded in an intricate network of reactions. The Michaelis-Menten Formalism does not produce a systematically structured approach for analysis of such complex systems.

The central assumptions of this formalism restrict its application to systems with independent rates that are linear functions of enzyme levels and activities. Moreover, the resulting formalism leads to *ad hoc* mathematical descriptions that are not easy to study analytically when there are large numbers of reactions.

A third formalism that differs significantly from these two is the *Power-Law Formalism* (Savageau, 1969*ab;* 1970; 1971*ab;* 1972). Its roots are in chemical and biochemical kinetics, organismal biology, and network theory (including sensitivity theory and Bode analysis). Relevant landmarks in these areas are summarized in Table 1. This formalism represents the interactions of a system in a structured fashion that facilitates analysis, and yet it retains the essential character of the underlying nonlinear processes. This formalism provided the basis for a theory of intact biochemical systems, which is now called Biochemical Systems Theory.

Component Representation within Biochemical Systems Theory

In Biochemical Systems Theory, all functional relationships, including rate laws, conservation relations, and other constraints are represented by a Taylor series in a logarithmic coordinate system. The conditions necessary for the existence of a valid Taylor series are well known (Thomas & Finney, 1982) and generally applicable to biochemical systems of interest. Each term in such a series brings about a progressively better approximation to the actual function, and at each stage the error in the representation can be measured. Approximation by the first two terms of the Taylor's series is particularly simple, and corresponds to the best linear representation in the logarithmic coordinate system.

Estimation of the parameter values reduces to the well-known procedure of linear regression (Savageau, 1972). In its simplest graphical interpretation, one determines the rate of a particular process with all the variables affecting the process held constant at their nominal steady-state values *in situ*. Then one systematically fixes one of the variables at different values about its nominal steady state and again determines the corresponding rate. When plotted in logarithmic coordinates the data might appear as shown in Fig. 1. An appropriate linear regression gives the best straight line passing through the nominal

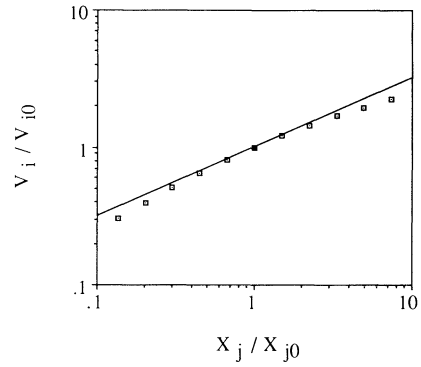


Figure 1. Estimation of component parameter values in Biochemical Systems Theory. The points represent experimental data and the curve is the best straight line through the nominal operating value $X_j = X_{j0}$.

operating point tangent to the experimentally-determined curve. The equation of this straight line is given by

$$\log V_i = \log \alpha_i + g_{ij} \log X_j \tag{1}$$

The slope g_{ij} corresponds to the conventional kinetic order and the intercept α_i to the conventional rate constant of chemical and biochemical kinetics. When this equation is converted to Cartesian coordinates by exponentiation, one obtains the power-law representation:

$$V_i = \alpha_i X_j^{g_{ij}} \tag{2}$$

In general there are several variables affecting any given process, and the above procedure must be repeated for each of them. The above equations then become

$$\log V_i = \log \alpha_i + \sum_j g_{ij} \log X_j \tag{3}$$

and

$$V_i = \alpha_i \prod_j X_j^{g_{ij}} \tag{4}$$

where

$$g_{ij} = \left(\frac{\partial V_i}{\partial X_j}\right)_0 \left(\frac{X_j}{V_i}\right)_0 \tag{5}$$

and

$$\alpha_i = V_{i0} \prod_j X_{j0}^{-g_{ij}} \tag{6}$$

Thus, there are two types of fundamental parameters in the power-law representation — kinetic orders and rate constants — each has a well-defined mathematical definition and a straightforward graphical interpretation (Savageau 1969*b*, 1971*a*, 1972).

System Representations within Biochemical Systems Theory

Although there were at least four obvious variants within Biochemical Systems Theory that were recognized from the beginning (see Voit & Savageau, 1987), and it is now clear that there are many more (Sorribas & Savageau, 1989*c*), comparisons on the basis of the operations characteristic of each variant have never been published. In this section we shall make such comparisons among the more important variants within Biochemical Systems Theory. The principal distinctions, as summarized in Fig. 2, are manifested at three levels. First, the Power-Law Formalism that underlies all of these approaches can be either made explicit or left implicit. Second, one can choose to aggregate elementary fluxes into net

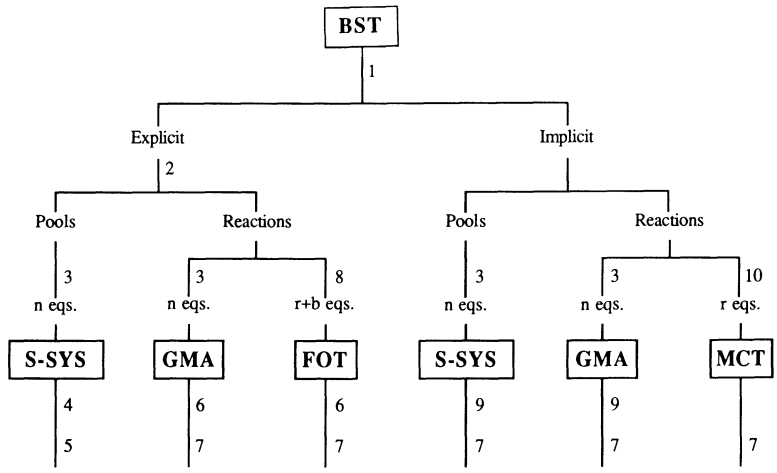


Figure 2. Variants within Biochemical Systems Theory (BST). See text for discussion and Sorribas & Savageau (1989*abc*) for additional detail. S-SYS = *S*-System; GMA = Generalized Mass Action; FOT = Flux-Oriented Theory; MCT = Metabolic Control Theory.

fluxes through pools or enzyme-catalysed reactions. Third, the systemic representation be formulated with minimal assumptions and the optimal number of equations known from network theory, or with more restrictive assumptions and more highly redundant equations. These distinctions in turn imply differences in the clarity, efficiency, power, and scope of the variants considered. To make the comparisons specific let us examine the system represented in Fig. 3, and to keep it simple let us follow only *one* of the equations through the major steps in the analysis of each variant.

Explicit S-System Representation within Biochemical Systems Theory. The major steps in this case are the following [see Sorribas & Savageau (1989*a*) for the full details]:

1. Formulate the component representations as described in the previous section.

2. Write Kirchhoff's node equation for each dependent variable of the system.

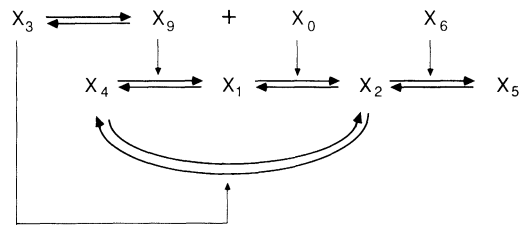


Figure 3. Enzyme-enzyme interactions and channelling of metabolic flux. X_4 and X_5 are independent metabolite concentrations; X_1 and X_2 are dependent metabolite concentrations; X_6, X_9 and X_0 are concentrations of "free" enzyme; X_3 is the concentration of the multienzyme complex; $X_7 = X_3 + X_9$ is the total concentration of the first enzyme; and $X_8 = X_3 + X_0$ is the total concentration of the second enzyme.

$$\frac{dX_2}{dt} = (v_{12} + v_{42}) - v_{25} \tag{7}$$

3. Aggregate elementary fluxes into net fluxes through pools, which allows the above equation to be written as

$$\frac{dX_2}{dt} = V_2 - V_{-2} \tag{8}$$

4. Each net rate law is replaced by the corresponding component representation in the Power-Law Formalism to generate the system representation. There is one power law for each variable that affects the rate law in question.

$$\frac{dX_2}{dt} = \alpha_2 X_1^{g_{21}} X_2^{g_{22}} X_3^{g_{23}} X_4^{g_{24}} X_0^{g_{20}} - \beta_2 X_2^{h_{22}} X_5^{h_{25}} X_6^{h_{26}} \tag{9}$$

With a little practice, this system representation in the Power-Law Formalism can be written immediately by inspection of the system diagram (e.g. Fig. 3). The system of differential equations then can be used to predict the dynamic behaviour of the system within a local region about its nominal operating point (Savageau, 1970; Voit *et al.*, 1989).

5. The equations characterizing the steady-state behaviour are obtained by setting the time derivatives to zero and transforming the resulting nonlinear algebraic equations into a set of linear algebraic equations.

$$g_{21}y_1 + a_{22}y_2 + g'_{23}y_3 = (b_2 - g_{24}y_4 + h_{25}y_5 + h_{26}y_6 - g_{28}y_8) \tag{10}$$

where $b_i = \log(\beta_i/\alpha_i)$, $a_{ij} = g_{ij} - h_{ij}$, and $y_i = \log X_i$.

6. The steady-state equations can be solved explicitly for each dependent variable

$$y_i = \sum_{j=1}^{3} M_{ij} b_j + \sum_{k=4}^{8} L_{ik} y_k \quad \text{for } i = 1, 2, 3 \tag{11}$$

provided $|\mathbf{A}| = a_{33}(a_{11}a_{22} + g_{21}h_{12}) \neq 0$. This solution allows one to predict the steady-state value of each dependent variable in terms of the values of the independent variables, kinetic orders, and rate constants (Savageau, 1969*b*).

7. Specific systemic responses also can be selected for emphasis by appropriate logarithmic differentiation with respect to change in an independent variable [e.g. Logarithmic Gain $L(X_2, X_6)$], a rate-constant parameter [e.g. Rate-Constant Sensitivity $S(X_2, \beta_2)$], or a kinetic-order parameter [e.g. Kinetic-Order Sensitivity $S(X_2, h_{22})$] (Savageau, 1971*ab*).

$$L(X_2, X_6) = \left(\frac{\partial X_2}{\partial X_6}\right)\left(\frac{X_6}{X_2}\right) = \frac{\partial y_2}{\partial y_6}, \quad L_{26} = a_{11}a_{33}h_{26}/|\mathbf{A}| \tag{12}$$

8. Step 7 can be repeated as often as desired or until all of the possibilities are exhausted.

Within the framework of the Power-Law Formalism the steady-state analysis using the explicit *S*-system representation is complete. Systemic properties are directly related to component properties in the nominal steady state by a simple matrix equation (Savageau, 1971*b*):

$$[\mathbf{L}] = -[\mathbf{A}]_d^{-1}[\mathbf{A}]_i \tag{13}$$

where the elements of [**L**] are the logarithmic gains (or response coefficients in Metabolic Control Theory) and the elements of [**A**] are the kinetic orders (or elasticities in Metabolic Control Theory) for the component reactions of the system. The subscripts *d* and *i* refer to kinetic orders with respect to dependent and independent variables respectively. This equation can be rewritten in a variety of manifestations as shown in Table 2 (Savageau & Sorribas, 1989). The influence of every independent concentration variable and every parameter value on every dependent variable of the system is accounted for. These influences are determined not only for the nominal steady state, but for all steady states in the local neighbourhood. The dynamic responses of the system within the local neighbourhood of the steady state also can be characterized.

Table 2. Complete Characterization of the Nominal Steady State within Biochemical Systems Theory[1]

Flux Variables			Concentration Variables		
Systemic Properties		Component Properties	Systemic Properties		Component Properties
$[\mathbf{L}(\mathbf{V}, \mathbf{X})]$	=	$[\mathbf{G}]_i - [\mathbf{G}]_d[\mathbf{A}]_d^{-1}[\mathbf{A}]_i$	$[\mathbf{L}(\mathbf{X}, \mathbf{X})]$	=	$-[\mathbf{A}]_d^{-1}[\mathbf{A}]_i$
$[\mathbf{S}(\mathbf{V}, \beta)]$	=	$[\mathbf{G}]_d[\mathbf{A}]_d^{-1}$	$[\mathbf{S}(\mathbf{X}, \beta)]$	=	$[\mathbf{A}]_d^{-1}$
$[\mathbf{S}(\mathbf{V}, \alpha)]$	=	$[\mathbf{I}] - [\mathbf{G}]_d[\mathbf{A}]_d^{-1}$	$[\mathbf{S}(\mathbf{X}, \alpha)]$	=	$-[\mathbf{A}]_d^{-1}$
$\{\mathbf{S}(\mathbf{V}, \mathbf{h})/\mathbf{h}\}$	=	$[\mathbf{G}]_d[\mathbf{A}]_d^{-1} \otimes \mathbf{y}]$	$\{\mathbf{S}(\mathbf{X}, \mathbf{h})/\mathbf{h}\}$	=	$[\mathbf{A}]_d^{-1} \otimes \mathbf{y}]$
$\{\mathbf{S}(\mathbf{V}, \mathbf{g})/\mathbf{g}\}$	=	$[\mathbf{I}] \otimes \mathbf{y}] - [\mathbf{G}]_d[\mathbf{A}]_d^{-1} \otimes \mathbf{y}]$	$\{\mathbf{S}(\mathbf{X}, \mathbf{g})/\mathbf{g}\}$	=	$-[\mathbf{A}]_d^{-1} \otimes \mathbf{y}]$

[1]See Savageau & Sorribas (1989).

Explicit Generalized-Mass-Action Representation within Biochemical Systems Theory. One of the obvious variants within Biochemical Systems Theory corresponds to aggregation of elementary fluxes into net fluxes through reactions and yields the Generalized-Mass-Action representation within Biochemical Systems Theory. The major steps in the analysis according to this variant are the following [see Sorribas & Savageau (1989*b*) for details]:

1. Formulate the component representations as described in the previous section.

2. Write Kirchhoff's node equation for each dependent variable of the system.

$$\frac{dX_2}{dt} = v_{12} + v_{42} - v_{25} \tag{14}$$

3. Aggregate elementary fluxes into net fluxes through reactions, which is actually assumed in the above notation, and then rewrite eqn. (14) using the convention adopted in Biochemical Systems Theory:

$$\frac{dX_2}{dt} = V_{21} + V_{22} - V_{-2} \tag{15}$$

4. Each net rate law is replaced by the corresponding component representation in the Power-Law Formalism to generate the system representation. There is one power law for each variable that affects the rate law in question.

$$\frac{dX_2}{dt} = \alpha_{21} X_1^{g211} X_2^{g221} X_0^{g201} + \alpha_{22} X_2^{g222} X_3^{g232} X_4^{g242} - \beta_2 X_2^{h22} X_5^{h25} X_6^{h26} \tag{16}$$

With a little practice, this system representation in the Power-Law Formalism can be written by inspection of the system diagram (e.g. Fig. 3). The system of differential equations then can be used to predict the dynamic behaviour of the system within a local region about its nominal operating point.

5. The nonlinear algebraic equations characterizing the steady-state behaviour are obtained by setting the time derivatives to zero.

$$0 = \alpha_{21} X_1^{g211} X_2^{g221} X_0^{g201} + \alpha_{22} X_2^{g222} X_3^{g232} X_4^{g242} - \beta_2 X_2^{h22} X_5^{h25} X_6^{h26} \tag{17}$$

In general, there is no explicit steady-state solution for this system of nonlinear equations. A different approach to characterizing the steady state must be followed at this point.

6. Specific systemic responses can be defined by explicit differentiation of the above equations with respect to an independent variable, a rate-constant parameter, or a kinetic-order parameter. For example, differentiation with respect to the enzyme level X_6 yields the following equation.

$$\begin{aligned} g211\left(\frac{v_{12}}{v_{25}}\right)\left(\frac{\partial X_1}{\partial X_6}\right)\left(\frac{X_6}{X_1}\right) + \left[g221\left(\frac{v_{12}}{v_{25}}\right) + g222\left(\frac{v_{42}}{v_{25}}\right) - h22\right]\left(\frac{\partial X_2}{\partial X_6}\right)\left(\frac{X_6}{X_2}\right) \\ + \left[g231\left(\frac{v_{12}}{v_{25}}\right) + g232\left(\frac{v_{42}}{v_{25}}\right)\right]\left(\frac{\partial X_3}{\partial X_6}\right)\left(\frac{X_6}{X_3}\right) = h26 \end{aligned} \tag{18}$$

The relative derivatives are recognized as the set of logarithmic gains with respect to X_6

$$g211\left(\frac{v_{12}}{v_{25}}\right)L_{16} + \left[g221\left(\frac{v_{12}}{v_{25}}\right) + g222\left(\frac{v_{42}}{v_{25}}\right) - h22\right]L_{26} + \left[g231\left(\frac{v_{12}}{v_{25}}\right) + g232\left(\frac{v_{42}}{v_{25}}\right)\right]L_{36} = h26 \tag{19}$$

and, since the coefficients preceding the logarithmic gains are readily seen to be the corresponding kinetic orders in the *S*-system representation, the equation can be written more simply as

$$g_{21}L_{16} + g_{22}L_{26} + g'_{23}L_{36} = h_{26} \tag{20}$$

7. Specific systemic responses can be determined by solving the linear equations obtained in the previous step. For example,

$$L_{26} = a_{11}a_{33}h_{26}/|\mathbf{A}| \tag{21}$$

provided $|\mathbf{A}| = a_{33}(a_{11}a_{22} + g_{21}h_{12}) \neq 0$

8. Steps 6 and 7 can be repeated as often as desired or until all of the possibilities are exhausted.

Comparison of Explicit S-System and Explicit Generalized-Mass-Action Representations. Note that the systemic factors — logarithmic gains [e.g. $L(X_2, X_6)$], rate-constant sensitivities [e.g. $S(X_2, \beta_2)$], and kinetic-order sensitivities [e.g.. $S(X_2, h_{22})$] — obtained with the explicit Generalized-Mass-Action variant are *identical* to the corresponding systemic factors obtained with the explicit *S*-system variant of Biochemical Systems Theory. Thus, they produce the same characterization of the nominal steady state. Because there is no explicit steady-state solution with the Generalized-Mass-Action representation, it is necessary to reverse the order of the operations in steps 6 and 7. This results in loss of information about the system and has important implications for predictions of behaviour away from the nominal steady state. One cannot obtain the systemic behaviour explicitly. In many cases one can predict systemic behaviour for numerically characterized systems; but, as demonstrated elsewhere (Sorribas & Savageau, 1989*b*), these predictions are generally less accurate than those made with the *S*-system representation. The analysis with the Generalized-Mass-Action representation also tends to be less efficient because one must repeatedly differentiate the set of steady-state equations and solve the resulting linear system, whereas with the *S*-system representation one solves a linear system once[1] and then differentiates the result once for each systemic factor of interest.

Explicit Representation within Flux-Oriented Theory. The Flux-Oriented Theory of Crabtree & Newsholme (1987) uses an explicit representation that can be considered a special case of the explicit Generalized-Mass-Action variant within Biochemical Systems Theory (see Sorribas & Savageau, 1989*b*, for details). In order to develop a deeper appreciation for the different approaches and how they relate to the general framework provided by the Power-Law Formalism, it is important to recall the principal differences between Flux-Oriented Theory and the explicit Generalized-Mass-Action variant. First, Flux-Oriented

[1]Perhaps it should be emphasized that solution of the linear system is the most time-consuming step in the mathematical analysis and that the difficulty of solution increases markedly with increasing number of variables in the system. Typically, solution time increases as a cubic or even factorial function of the number of variables, depending on the method of solution. Consequently, it is advisable to minimize the number of variables in the representation, especially when dealing with large biochemical systems. In contrast to the difficulty of solution by matrix inversion, the effort necessary to define aggregate parameters before solution and then to substitute those definitions back into the resulting solution is negligible.

Theory differs in the use of a non-standard matrix notation that makes analysis and interpretation somewhat more difficult, at least for larger systems. Second, it makes a number of restrictive assumptions — linear and independent variation in rates with variation in enzyme levels, reactions not in equilibrium, etc. — that cannot be considered generally valid. Third, it formulates the system representation in a non-optimal fashion that increases the difficulty of solving the resulting equations.

In particular, it is instructive to compare the enumeration of equations for the explicit Generalized-Mass-Action variant within Biochemical Systems Theory, which uses the standard enumeration in well-developed network theories, to that in Flux-Oriented Theory. In general, the combination of rate laws and Kirchhoff's flux laws for a given system will generate $r + n$ equations in $r + n$ variables, where r is the number of reactions and n is the number of dependent concentrations. In presenting Flux-Oriented Theory, Crabtree & Newsholme (1987) define b as the number of branch points in the system and have reduced the above numbers to $r + b$ equations in $r + b$ unknowns by using Kirchhoff's flux equations at the simpler, non-branching, nodes of the system to eliminate one of a pair of equivalent flux variables. These numbers are reduced still further — to the minimum, n equations in n unknowns — in the explicit Generalized-Mass-Action variant within Biochemical Systems Theory by substituting the appropriate rate laws into Kirchhoff's flux equation for *each* dependent concentration variable. This is standard technique in network theory (see also the previous footnote).

For the system in Fig. 3, one can assume equilibrium for the enzyme-enzyme association/dissociation and eliminate these processes from consideration in steady state, and then $r = 4$, $n = 2$ and $b = 1$. The full system is 6th order, the partially reduced system in Flux-Oriented Theory is 5th order, and the fully reduced system in the explicit Generalized-Mass-Action variant is 2nd order. Thus, the explicit Generalized-Mass-Action variant within Biochemical Systems Theory, when compared with the special case of Flux-Oriented Theory, makes less restrictive assumptions, uses a more efficient reduction of the initial $r + n$ equations, and provides a more systematic representation for analysis of the system.

Implicit Generalized-Mass-Action Representation within Biochemical Systems Theory. In contrast to the previous variants, which develop the component representation within the Power-Law Formalism first and then use it explicitly, another class of variants uses the Power-Law Formalism implicitly. As an example of this class let us consider the implicit Generalized-Mass-Action representation within Biochemical Systems Theory. The major steps in the analysis according to this variant are the following [see Sorribas & Savageau (1989*b*) for details].

1. Write Kirchhoff's node equation for each dependent variable of the system.

$$\frac{dX_2}{dt} = v_{12} + v_{42} - v_{25} \tag{22}$$

2. Aggregate elementary fluxes into net fluxes through reactions, and rewrite the above

equation as

$$\frac{dX_2}{dt} = V_{21} + V_{22} - V_{-2} \tag{23}$$

3. The nonlinear algebraic equations characterizing the steady-state behaviour are obtained by setting the time derivatives to zero:

$$0 = V_{21} + V_{22} - V_{-2} \tag{24}$$

In this form of the steady-state equations no explicit steady-state solution is possible; nor can a steady-state solution be obtained numerically without specifying some explicit form for the rate laws. A different approach to characterizing the steady state must be followed at this point.

4. Implicit logarithmic differentiation of each rate law with respect to each X variable, while holding all other X variables fixed, defines one class of component parameters within Biochemical Systems Theory — the kinetic orders.

5. Implicit logarithmic differentiation of each X variable with respect to a specific independent X variable, while holding all other independent X variables fixed, defines one class of systemic properties within Biochemical Systems Theory — the logarithmic gains.

6. The sequential execution of steps 4 and 5 in the chain rule of differentiation leads to an implicit systemic representation within Biochemical Systems Theory. For example, differentiation with respect to the independent variable X_6 yields the following equation:

$$\begin{aligned}&\frac{\partial v_{12}}{\partial X_1}\frac{X_1}{v_{12}}\frac{v_{12}}{v_{25}}\frac{\partial X_1 X_6}{\partial X_6 X_1} + \frac{\partial v_{12}}{\partial X_2}\frac{X_2}{v_{12}}\frac{v_{12}}{v_{25}}\frac{\partial X_2 X_6}{\partial X_6 X_2} + \frac{\partial v_{12}}{\partial X_3}\frac{X_3}{v_{12}}\frac{v_{12}}{v_{25}}\frac{\partial X_3 X_6}{\partial X_6 X_3} \\ &+ \frac{\partial v_{42}}{\partial X_2}\frac{X_2}{v_{42}}\frac{v_{42}}{v_{25}}\frac{\partial X_2 X_6}{\partial X_6 X_2} + \frac{\partial v_{42}}{\partial X_3}\frac{X_3}{v_{42}}\frac{v_{42}}{v_{25}}\frac{\partial X_3 X_6}{\partial X_6 X_3} - \frac{\partial v_{25}}{\partial X_2}\frac{X_2}{v_{25}}\frac{\partial X_2 X_6}{\partial X_6 X_2} = \frac{\partial v_{25}}{\partial X_6}\frac{X_6}{v_{25}}\end{aligned} \tag{25}$$

The logarithmic or relative derivatives of the rate laws with respect to the X variables are recognized as the appropriate kinetic orders, and the logarithmic derivatives of the X variables with respect to X_6 are identified as the set of logarithmic gains.

$$g_{211}\left(\frac{v_{12}}{v_{25}}\right)L_{16} + \left[g_{221}\left(\frac{v_{12}}{v_{25}}\right) + g_{222}\left(\frac{v_{42}}{v_{25}}\right) - h_{22}\right]L_{26} + \left[g_{231}\left(\frac{v_{12}}{v_{25}}\right) + g_{232}\left(\frac{v_{42}}{v_{25}}\right)\right]L_{36} = h_{26} \tag{26}$$

Since the coefficients preceding the logarithmic gains are readily seen to be the corresponding kinetic orders in the S-system representation, the equation can be written more simply as

$$g_{21}L_{16} + g_{22}L_{26} + g'_{23}L_{36} = h_{26} \tag{27}$$

7. Specific systemic responses, i.e. logarithmic gains, can be determined by solving the

linear equations obtained in the previous step. For example,

$$L_{26} = a_{11}a_{33}h_{26}/|\mathbf{A}| \tag{28}$$

provided $|\mathbf{A}| = a_{33}(a_{11}a_{22} + g_{21}h_{12}) \neq 0$

8. Steps 4, 5, 6, and 7 can be repeated as often as desired or until one exhausts the possibilities.

Comparison of Implicit Generalized-Mass-Action with Explicit S-System and Explicit Generalized-Mass-Action Representations. Note that the systemic properties — logarithmic gains [e.g. $L(X_2, X_6)$] — obtained with the implicit Generalized-Mass-Action variant are *identical* to the corresponding systemic properties obtained with the explicit *S*-system and explicit Generalized-Mass-Action variants within Biochemical Systems Theory. That is, the implicit Generalized-Mass-Action representation leads to the same characterization of the nominal steady state with respect to changes in the independent variables. However, because there is no explicit steady-state solution with the implicit Generalized-Mass-Action representation, it is necessary to reorder the steps in the analysis, which results in loss of information about the system. The loss is more profound in this case than it is in the case of the explicit Generalized-Mass-Action representation. Determination of systemic properties involving rate constants and kinetic orders requires that the underlying formalism be explicit; hence, these properties cannot be obtained by an implicit approach. An implicit approach allows neither an explicit symbolic solution nor a numerical solution of the systemic behaviour in terms of the underlying parameters and independent variables. As a consequence one cannot determine the dynamic behaviour of the system, nor can one determine the steady-state behaviour away from the nominal operating point, which is in contrast to the situation with explicit approaches.

The partial analysis possible with the implicit Generalized-Mass-Action representation tends to be less efficient because one must repeatedly differentiate the set of steady-state equations and solve the resulting linear system, whereas with the explicit *S*-system representation one solves a linear system once and then differentiates the result once for each systemic property of interest (see also the previous footnote). Moreover, differentiation in the implicit approach is more complicated, and one must be careful to distinguish the different *kinds* of differentiation that are involved; this is due to a re-derivation of the component representation by logarithmic differentiation each time one carries out an implicit analysis. In the explicit use of the Power-Law Formalism, the differentiation steps used to derive the component representation have been given in general and need not be repeated for each analysis, and thus these differentiation steps are well separated from the subsequent differentiation steps used to emphasize specific systemic responses.

Implicit Representation within Metabolic Control Theory. Metabolic Control Theory is based on a particular set of constraint equations, called summation and connectivity theorems (see Kacser & Burns, 1973; Heinrich & Rapoport, 1974; Kacser & Porteous, 1987

and references therein; also Chapter 3 by Porteous in this book), that on the surface might appear to be quite different from those of the other variants discussed above. However, it has been shown that, from a fundamental point of view, Metabolic Control Theory is a special case of the implicit Generalized-Mass-Action variant within Biochemical Systems Theory (Savageau *et al.*, 1987*ab;* Sorribas & Savageau, 1989*bc;* Savageau & Sorribas, 1989). The most difficult point to understand from the perspective of Metabolic Control Theory seems to be the relationship between the summation and connectivity theorems and the general framework of the Power-Law Formalism. Recent efforts aimed at developing a more general basis for Metabolic Control Theory [see Chapters 7 (Reder), 8 (Mazat and Reder), 11 (Canela, Cascante and Franco), 30 (Giersch, Steffen and Lämmel), and others in this book] provide additional perspectives within Metabolic Control Theory that may make it easier to discern this relationship.

In particular, a generalization that removes some of the restrictions of Metabolic Control Theory has been presented by Cascante *et al.* (1989*ab*). These authors base their approach on sensitivity theory rather than the summation and connectivity theorems of Metabolic Control Theory, but continue to leave the Power-Law Formalism implicit. It is clear from this approach that Metabolic Control Theory, when properly generalized, is a special case of the implicit Generalized-Mass-Action variant within Biochemical Systems Theory. The approach of Cascante *et al.* (1989*ab*) includes the generalization presented by Kacser, Sauro and Acerenza in Chapter 20 of this book. The latter approach also removes some of the restrictions inherent in Metabolic Control Theory, although it continues to be based on summation and connectivity theorems, as well as implicit use of the Power-Law Formalism. One of the restrictions that remain in this case is the requirement that the reaction rates be independent of one another.

The generalized relationships between systemic and component properties presented in these recent papers are equivalent to eqn. (13), which was obtained using the explicit *S*-system representation (Savageau, 1971*b*). However, the derivation of this equation (for additional details, see Sorribas & Savageau, 1989*a*) does not require the assumption that reaction rates be independent of one another, and therefore eqn. (13) is valid even under conditions where the summation and connectivity relationships are invalid (see also Sorribas & Savageau, 1989*b*).

Metabolic Control Theory also formulates the system representation in a non-optimal fashion that increases the difficulty of solving the resulting equations. This can be seen by contrasting the enumeration of equations in the implicit Generalized-Mass-Action variant with that in Metabolic Control Theory. Since the implicit Generalized-Mass-Action variant is closely related to the explicit Generalized-Mass-Action variant, the implicit representation also involves n equations in n unknowns. On the other hand, solution in Metabolic Control Theory involves the summation and connectivity relationships, which implies r equations in r unknowns, where r is the number of reactions and $r > n$ [e.g. see Fell & Sauro (1985)]. Thus, the size of the system that must be solved simultaneously is larger than the minimum n determined by standard criteria from network topology. For the case in Fig. 3, the difference is $r = 4$ versus $n = 2$, provided one eliminates the enzyme-enzyme interactions and assumes that X_3, X_9, and X_0 are linearly independent enzyme activities so that Metabolic Control Theory can be applied (see also the footnote above).

Summary of Comparisons. The results of detailed comparisons based on objective criteria show that all of the approaches examined (and many others that cannot be considered here: see Voit & Savageau, 1987; Sorribas & Savageau, 1989*c*; also Chapter 5 by Voit in this book) can be considered variants within Biochemical Systems Theory. In no case does any variant provide more information than is provided by the *S*-system representation. The other variants may provide an alternative view of control in metabolic systems, but they do *not* provide a separate theory. All of the variants are similar to the extent that they involve the same experimental measurements, the same definitions of component parameters and systemic properties, the same steps in the analysis, and the same results when applied to the same situation. Their differences are manifested at three levels, as indicated in Fig. 2. Differences at each of these levels have many implications, some of which are summarized in Table 3. The inclusiveness or scope of each of these variants — which for the most part parallels their power, efficiency, and clarity — is readily apparent.

Table 3. Summary of Results

Features of the theory	Variants of Biochemical Systems Theory					
	Implicit			Explicit		
	MCT[1]	GMA[2]	*S*-System	FOT[3]	GMA[2]	*S*-System
Least restrictive assumptions		+	+		+	+
Systematic notation	±	+	+		+	+
Efficient reduction of equation set		+	+		+	+
Effect of independent variables on steady state	+	+	+	+	+	+
Effect of kinetic orders on steady state				+	+	+
Effect of rate constants on steady state					+	+
Component & systemic derivatives unmixed				+	+	+
Full power-law notation					+	+
Efficient solution of systemic properties				±	±	+
Efficient numerical solution of steady states					±	+
Efficient numerical solution of dynamics					±	+
Wide range of accuracy					±	+
Explicit steady-state solutions						+

[1]Metabolic Control Theory; [2]Generalized Mass Action; [3]Flux-Oriented Theory

Applications

If one were to identify the most outstanding characteristic of the explicit *S*-system representation within Biochemical Systems Theory, it would be its ability to yield explicit steady-state solutions in symbolic form (Savageau, 1969*b*). Such solutions are rare for complex nonlinear systems, but when they exist, important consequences follow. The existence of

symbolic solutions for different systems being compared allows one to equate specific systemic responses while exploring the implications of alternative values for their component parameters. This provides the mathematical equivalent of a "well-controlled" experiment (Savageau, 1972, 1976; Irvine & Savageau, 1985). Such analyses with symbolic solutions often lead to very general conclusions that are valid for entire classes of systems, independent of the particular numerical values associated with the parameters of specific systems (e.g. see Savageau, 1976; Savageau & Voit, 1987). Such analyses have succeeded where others requiring numerical values have not, because numerical values often are unknown and in some cases are difficult or impractical to obtain experimentally. Although symbolic analysis generally is more difficult, the rewards are correspondingly greater.

Table 4. Applications that have used the Explicit *S*-System representation of Biochemical Systems Theory[1]

Applications in Biology	
Unbranched biosynthetic pathways	Growth
Branched biosynthetic pathways	Development
Amphibolic pathways	Regeneration
Cascade mechanisms	Immune networks
Inducible operons	Fermentation
Repressible operons	Epidemiology
Gene circuits	Population dynamics
Applications in Other Areas	
Dynamical systems	Economics
Numerical analysis	Statistics
Chemical kinetics	

[1]For references see Savageau & Voit (1987)

Biochemical Systems Theory also has been applied to many specific systems. From the beginning it has been used to predict cellular and organismal responses to change in environmental conditions and in underlying molecular determinants, and to elucidate the design principles of biological systems. The utility of the *S*-system representation within Biochemical Systems Theory has been recognized and applied in many other areas as well, including a number of areas outside biology (Table 4). In many cases these applications have led to more specialized theories with strong predictive capabilities (e.g. see Savageau & Voit, 1987; Savageau, 1989). A more detailed review of applications is outside the scope of this chapter; the interested reader is encouraged to consult some of the recent reviews cited above.

Recent Developments within Biochemical Systems Theory

Like any other mathematical representation of phenomena in the real world, the variants of Biochemical Systems Theory have their limitations. The most significant may be the problem of accuracy and the problem of dynamic solution. In the remainder of this section I

shall comment briefly on these issues and on emerging opportunities for advancements within the Power-Law Formalism that address these limitations. A more detailed treatment of the accuracy question in given by Voit in Chapter 5 of this book.

The S-System is a Canonical Nonlinear Representation. The first and most characteristic limitation of Biochemical Systems Theory, like that of all local representations, is the range of variation in concentration over which the representation is valid. Although this range is broader for the explicit *S*-system variant within Biochemical Systems Theory than for other well-known local representations (Voit & Savageau, 1987), and although it is comparable to the ranges that are exhibited experimentally in biochemical systems (Sorribas & Savageau, 1989*a*), it nonetheless is a potential problem for some systems. For instance, if the response of a system is non-monotonic over a given range of variation, a single power-law representation may not be satisfactory.

What are the alternatives when one encounters a situation like this? One obvious choice is to fall back on a more complex nonlinear strategy, such as representation by rational functions. This may provide acceptable numerical descriptions for specific systems, but its limitations for revealing more general, class-specific properties of biochemical systems are well known. Another choice, for which there is ample precedent, is "piecewise representation". One can subdivide the actual range of operation into smaller ranges within which the behaviour is monotonic and represented accurately by separate power-law functions. This is a straightforward analogy to the well-known methods for piecewise linear representation. Again, this may lead to acceptable numerical descriptions for specific systems, but by introducing an *ad hoc* subdivision into the theory one loses its coherent and general characteristics.

An alternative to these *ad hoc* approaches has recently become available as a result of developments within the Power-Law Formalism itself (see Savageau & Voit, 1987). It is now possible to remain within the Power-Law Formalism, and by introducing additional variables, improve the range of representation or in fact achieve an *exact* representation for the nonlinear functions likely to be encountered in any biochemical system. In essence, one can *recast* a nonlinear system exactly as an *S*-system, which is now seen to be a canonical nonlinear representation. This approach has the advantage of staying within a unified theoretical framework. It also means that the powerful methods already developed to solve differential equations in the Power-Law Formalism can be applied here as well.

ESSYNS — State-of-the-Art Programs for Analysis of Biochemical Systems. The second major limitation, following these problems of accurate representation, is in the methods for solving the full dynamic problem in Biochemical Systems Theory. Unlike the steady-state solution, which is straightforward because it represents a linear problem when transformed into logarithmic coordinates, the dynamic solution involves nonlinear differential equations. Dynamics are a fundamental problem for any realistic nonlinear formalism. In general, one cannot obtain dynamic solutions except for specific numerical cases and, even for these, the available methods are often unsatisfactory in terms of reliability, accuracy, and efficiency (Press *et al.*, 1986). Recent developments within the Power-Law Formalism have led to significant advances in methods for numerical solution of nonlinear dynamics as well (Irvine

& Savageau, 1990). As a result dynamic solutions can now be obtained one to two orders of magnitude faster and with greater reliability and accuracy (Irvine & Savageau, 1990) than is possible with other methods.

This is routinely accomplished with a menu-driven user-friendly program that has been under development since the late 1960s (see Irvine & Savageau, 1990). The current version, called ESSYNS (for Evaluation and Simulation of Synergistic Systems), runs on an IBM PC, AT, PS/2 or a compatible computer. The program includes state-of-the-art methods for solving differential equations (Irvine & Savageau, 1990), the complete steady-state analysis described above, graphical presentation and analysis, as well as data management facilities (Voit *et al.*, 1989). Information about how to obtain this and other programs may be found in Appendix B to this book.

Conclusions

The Power-Law Formalism does not simply refer to the common power-law function, which has been used in biology since the time of Galileo, but to a formal mathematical structure that has been systematically elaborated over the past 20 years. Like other formal mathematical languages (e.g. the Linear Formalism) it includes precise definitions, systematic notation, strategies for representation, strategies for determination of accuracy, strategies for estimation of parameter values, existence theorems, methods of analytical solution, and methods of computer-assisted analysis. This formalism provides the basis for Biochemical Systems Theory. Different forms of representation within this theory were recognized from the beginning. However, once the advantages of aggregation into net rate laws for synthesis and degradation (S-system representation) were discovered, this became the focus for most of the early work. Others have chosen to focus on different variants within Biochemical Systems Theory. As the evidence presented in this chapter demonstrates, the explicit S-system variant has obvious advantages since there is some loss of information, accuracy, efficiency, and simplicity with each of the other variants.

Methods for making well-controlled comparisons of alternative designs have been developed within Biochemical Systems Theory and applied to several classes of biochemical systems including metabolic pathways, metabolic control mechanisms, cascade mechanisms, molecular mechanisms of gene expression, regulatory gene circuits, network regulation of the immune response, and plant growth. In many cases these applications have led to more specialized theories with strong predictive capabilities; examples include a theory of design for metabolic control mechanisms, a demand theory of gene regulation, and a theory of gene circuits. Specific predictions of these theories have been confirmed by experimental results from a number of independent laboratories.

Finally, recent developments within Biochemical Systems Theory have led to significant advances in the state of the art for computer-assisted analysis of biochemical systems and to the discovery that the explicit S-system representation provides a canonical nonlinear form into which rather arbitrary nonlinear functions can be recast exactly. These developments demonstrate the potential for further growth of Biochemical Systems Theory and the underlying Power-Law Formalism on which it is based.

REFERENCES

Cascante, M., Franco, R. & Canela, E. I. (1989*a*) *Math. Biosci. 94,* 271-288

Cascante, M., Franco, R. & Canela, E. I. (1989*b*) *Math. Biosci. 94,* 289-309

Crabtree, B. & Newsholme, E. A. (1987) *Biochem. J. 247,* 113-120

Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561

Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95

Irvine, D. H. & Savageau, M. A. (1985) *J. Immunol. 134,* 2100-2116

Irvine, D. H. & Savageau, M. A. (1990) *SIAM J. Numerical Anal.* in press

Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104

Kacser, H. & Porteous, J. W. (1987) *Trends Biochem. Sci. 12,* 5-14

Press, W. H., Flannery, B. P., Teukolsky, S. A. & Vetterling, W. T. (1986) *Numerical Recipes: the Art of Scientific Computing,* Cambridge University Press, Cambridge

Savageau, M. A. (1969*a*) *J. Theor. Biol. 25,* 365-369

Savageau, M. A. (1969*b*) *J. Theor. Biol. 25,* 370-379

Savageau, M. A. (1970) *J. Theor. Biol. 26,* 215-226

Savageau, M. A. (1971*a*) *Nature 229,* 542-544

Savageau, M. A. (1971*b*) *Arch. Biochem. Biophys. 145,* 612-621

Savageau, M. A. (1972) *Curr. Top. Cell. Reg. 6,* 63-130

Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts

Savageau, M. A. (1989) in *Theoretical Biology — Epigenetic and Evolutionary Order* (Goodwin, B. C. & Saunders, P. T., eds.) pp 42-66, Edinburgh University Press, Edinburgh

Savageau, M. A. & Sorribas, A. (1989) *J. Theor. Biol. 141,* 93-114

Savageau, M. A. & Voit, E. O. (1987) *Math. Biosci. 87,* 83-115

Savageau, M. A., Voit, E. O. & Irvine, D. H. (1987*a*) *Math. Biosci. 86,* 127-145

Savageau, M. A., Voit, E. O. & Irvine, D. H. (1987*b*) *Math. Biosci. 86,* 147-169

Sorribas, A. & Savageau, M. A. (1989*a*) *Math. Biosci. 94,* 161-193

Sorribas, A. & Savageau, M. A. (1989*b*) *Math. Biosci. 94,* 195-238

Sorribas, A. & Savageau, M. A. (1989*c*) *Math. Biosci. 94,* 239-269

Thomas, G. B., Jr. & Finney, R. L. (1982) *Calculus and Analytical Geometry,* 5th edn., Addison-Wesley, Reading, Massachusetts

Voit, E. O. & Savageau, M. A. (1987) *Biochemistry 26,* 6869-6880

Voit, E. O., Irvine, D. H. & Savageau, M. A. (1989) *The User's Guide to* ESSYNS, Medical University of South Carolina Press, Charleston

Chapter 5

Comparison of Accuracy of Alternative Models for Biochemical Pathways

EBERHARD O. VOIT

DURING the past two decades, three new theories have been developed for the representation and analysis of biochemical phenomena: *Biochemical Systems Theory,* originated by Savageau (1969*ab,* 1970, 1971, 1972), *Metabolic Control Theory,* originated by Kacser & Burns (1973) and Heinrich & Rapoport (1974, 1975), and the theory originated by Crabtree & Newsholme (1978, 1985, 1987), which I shall call *Flux-Oriented Theory* (cf. Sorribas & Savageau, 1989*b*). All three theories have the ultimate goal to yield insight into the function and regulation of biochemical systems. In particular, they all intend to answer the question of how component and global properties are related to each other or, in other words, how the function of an integrated biochemical system can be deduced from kinetic observations of the component parts. Comparisons on the basis of the underlying theory (Savageau, Voit & Irvine, 1987*ab*), of results from application to the same systems (Sorribas & Savageau, 1989*abc*), and of the specific operations involved in the execution of an analysis (as described by Savageau in Chapter 4 of this book) all have shown that these three theories are related variants based on the *Power-Law Formalism*, even though some of the specific aims and applications of each approach may appear to be different.

One major distinction between Biochemical Systems Theory on the one hand and Metabolic Control Theory and Flux-Oriented Theory on the other hand is the strategy of aggregation by which branch-points of biochemical networks are represented. In Biochemical Systems Theory the fluxes entering a metabolic pool through each of a set of converging reactions are aggregated to form the net rate of production of that metabolite; similarly, the fluxes leaving a metabolic pool through each of a set of diverging reactions are aggregated to form the net rate of removal of that metabolite. By contrast, in Metabolic Control Theory and Flux-Oriented Theory forward and reverse fluxes through each reaction are aggregated to form the net rate of conversion for that reaction. The strategy of aggregation emphasized in Biochemical Systems Theory leads to a mathematical representation involving the difference between two products of power-law functions, which is known as

Eberhard O. Voit ● Department of Biometry, Medical University of South Carolina, Charleston, South Carolina 29425-2503, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

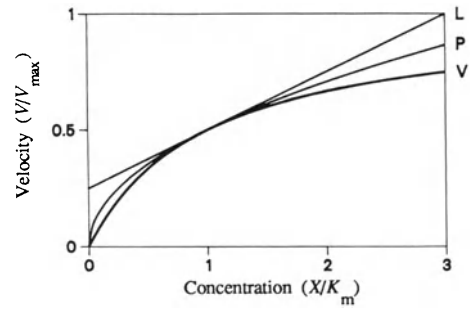


Figure 1. In Cartesian coordinates, the power-law representation (P) is clearly more appropriate than the linear representation (L) when a hyperbolic rate law (V) is studied over a wide range of concentrations.

an *S*-system. The strategy of aggregation in Metabolic Control Theory and Flux-Oriented Theory leads to a mathematical representation involving sums and differences of products of power-law functions, which is known as a *Generalized Mass Action* system.

In this chapter I shall examine the consequences of this difference for the accuracy of representation of biochemical systems. First, a brief review of the importance of accuracy will be given. Second, the accuracy of alternative representations for simple diverging and converging pathways will be compared. Third, I shall consider an unbranched amphibolic pathway. Finally, I shall show how the previous results for simpler systems translate into similar differences in accuracy for more complicated networks. The results show that the strategy of aggregating fluxes into two net rates, one for production and one for removal, yields more accurate representations than the alternative strategy involving power-law functions or linear representations.

Importance of Accuracy

Linear representations are typically the simplest. They can be derived in a straightforward fashion, and many powerful methods are available for characterizing most aspects of linear systems. Hence, one must ask whether it is really necessary to employ a more complex formalism for representing biochemical systems. The central issue is accuracy.

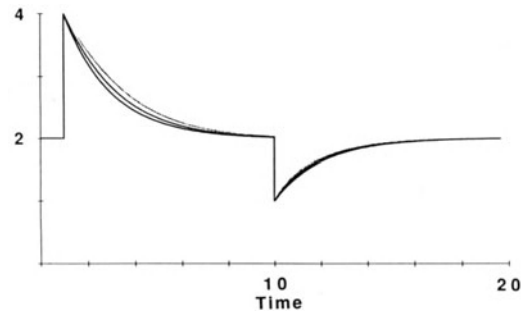


Figure 2. Linear (top curve) and power-law (middle curve) representations of individual rates of Michaelis-Menten type (bottom curve) both produce quite accurate dynamic responses to perturbations.

One would have to admit that linear models are often not very accurate representations of nonlinear phenomena (cf. Fig. 1). However, if only infinitesimal changes at steady state are of interest, then the accuracy of a linear model is sufficient and, in fact, no worse than that of any other representation. This is guaranteed by Taylor's theorem. Even for appreciable perturbations about the operating point, linear representations of individual reaction rates can be quite accurate and, thus, linear representations in many cases can be considered appropriate (cf. Fig. 2).

Nevertheless, the representation selected for Biochemical Systems Theory, Metabolic Control Theory and Flux-Oriented Theory is based on the relative derivative, which corresponds to linearization in a logarithmic space and, thus, to a non-linear representation. In Metabolic Control Theory and Flux-Oriented Theory, the linear representation in logarithmic space was motivated solely by the fact that relative derivatives are independent of scale. Furthermore, since these two theories only consider infinitesimal changes at steady state, questions of accuracy and dynamics were never asked. Biochemical Systems Theory, on the other hand, was developed to represent dynamical aspects as well as to facilitate the analysis of steady-state properties, and, hence, the question of accuracy had to be addressed from the beginning.

Among the various representations within the Power-Law Formalism, which result from different strategies for aggregating fluxes, the *S*-system was selected for development of Biochemical Systems Theory because it has a number of advantages over other representations. For example, this form allows direct analytical evaluation of steady-state properties such as existence and local stability of a steady state (Savageau, 1969*b*, 1975). Unlike linear representations, which also permit direct analytical evaluation, this non-linear approach is general enough to capture global features typical of biological systems, such as saturation, synergism, and stable oscillations.

It also was observed from the beginning that this representation produces results that are accurate over quite a wide range of variation in the system variables. This original impression was investigated during the development of Biochemical Systems Theory in different ways (e.g., Savageau, 1969*b*, 1972; 1976; 1979; Voit & Savageau, 1987; Sorribas & Savageau, 1989*abc*) and led to a growing body of evidence confirming that the Power-Law Formalism represents both steady-state and dynamical aspects of biological systems with surprising accuracy.

It is obvious that accuracy of representation is crucial for dynamical behaviour. But even if one is not explicitly interested in the dynamical aspects of a system, the question of accuracy must be addressed. Infinitesimal changes cannot be measured experimentally, and consequently, validation of any analysis must involve perturbations that are larger than the experimental error with which one can make a measurement. This is an intrinsic problem that is often side-stepped with the argument that the experimental perturbations are "small". However, in most cases no criterion is presented to decide whether "small" is small enough. Furthermore, even for small perturbations that are well within the normal ranges of biochemical systems accuracy of representation can be critical. If the system is sensitive to small variations in some of its parameter values and the mathematical representation does not capture the system behaviour with sufficient accuracy, even small perturbations can lead to considerably distorted results.

Since accuracy is of fundamental importance for the analysis of dynamical responses, as well as for experimental characterization of steady-state behaviour, it is necessary to evaluate the accuracy of alternative representations for biochemical pathways. In what follows I shall consider linear representations and power-law representations that are based on either *S*-systems (that are the focus of Biochemical Systems Theory) or Generalized Mass Action systems (that underlie Metabolic Control Theory and Flux-Oriented Theory).

The comparisons will focus on pathways described with Michaelis-Menten rate laws. However, because of the properties of the power-law approximation qualitatively similar results can be expected for other hyperbolic rate laws. The results may not apply to every other imaginable rate law, but the same types of comparisons have been performed for Hill rate laws, which are sigmoid and thus essentially different from Michaelis-Menten kinetics, and have yielded qualitatively similar results to those obtained here for Michaelis-Menten rate laws (cf. Voit & Savageau, 1987).

Comparison of S-system and Generalized Mass Action Representations for Branched Pathways

In this section, accuracy of representation will be determined for simple pathways composed of essentially irreversible reactions, which may be considered the fundamental components of more complex biochemical systems. Unbranched chains of such reactions are represented in exactly the same way by *S*-systems and Generalized Mass Action-systems and thus with respect to accuracy these two representations are equal. The differences show up when one considers diverging and converging pathways, for which the paradigms are illustrated in Figs. 3-4.

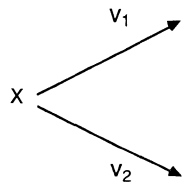


Figure 3. Simple diverging pathway.

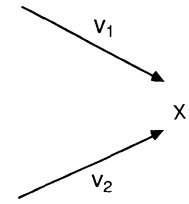


Figure 4. Simple converging pathway.

In the *S*-system representation all fluxes diverging from a node are aggregated to give a single net rate of loss for the branch-point metabolite, whereas in the Generalized Mass Action representation the fluxes are aggregated to give a separate rate of loss through each reaction. Similarly, in the *S*-system representation all fluxes converging to a node are aggregated to give a single net rate of production for the branch-point metabolite, whereas in the Generalized Mass Action representation production is described as a sum of separate rates derived by aggregation of fluxes through each reaction.

To the uninitiated, the form of aggregation found in the *S*-system representation sometimes seems unnatural because the representations for two processes are mathematically combined into one. The major argument is that there is deviation of mass balance at a branching node as soon as the system moves from the steady-state operating point: For instance, in a diverging pathway the sum of the two power laws representing synthesis of the products is different from the aggregated power-law term representing degradation of the branch-point substrate. While this is a correct observation, one has to recall that all representations of biochemical pathways are approximations with associated errors, and one has to take into account the advantages as well as the disadvantages of each representation to make a balanced assessment.

Comparing *S*-system representations with Generalized Mass Action system representations, one can enumerate several factors that counteract the initial concern with aggregation into net fluxes through pools. (i) One should note first that at the steady-state operating point the two representations give identical results, and mass balance as well as the individual fluxes themselves are accurately represented; thus, over the range of interest expressed in Metabolic Control Theory, the strategy of aggregation is not an issue. (ii) In the analysis of behaviour away form the steady-state operating value, the aggregated term can always be disaggregated, and no information concerning the individual fluxes is lost (cf. Sorribas & Savageau, 1989*a*). (iii) The aggregate rate of synthesis or degradation is no more difficult to measure experimentally than the individual rates of synthesis or degradation: one simply measures the change in concentration of the branch-point metabolite as one would for a metabolite that is not at a branch-point. (iv) The aggregation leading to the *S*-system representation allows general analytical evaluations, whereas that leading to the Generalized Mass Action representation allows only numerical evaluations; obviously, specific numerical results seldom provide the insight, generality, and power of prediction that analytical solutions offer. (v) Finally, we shall see in the following sections that the aggregation strategy associated with the *S*-system improves the accuracy of representation. That is, although there are deviations from mass balance at the branch-points, the individual fluxes and concentrations themselves are typically represented with greater accuracy.

Diverging Pathways. In a diverging pathway (Fig. 3), the branch-point substrate X is utilized via two different routes. Represented with Michaelis-Menten rate laws, the rate of utilization is

$$V = v_1 + v_2 = \frac{V_{max1} X}{K_{m1} + X} + \frac{V_{max2} X}{K_{m2} + X}$$

with V_{max} and K_m representing maximum velocity and Michaelis parameter, respectively. The corresponding *S*-system representation, designated by the subscript S, is based on the total rate V and consists of a single term:

$$V_S = \beta X^h$$

where the kinetic order h is defined as the logarithmic derivative of V at an operating point X_0:

$$h = \left(\frac{\partial V}{\partial X}\right)\left(\frac{X}{V}\right) = \frac{V_{max1} K_{m1}(K_{m2} + X_0)^2 + V_{max2} K_{m2}(K_{m1} + X_0)^2}{(K_{m1} + X_0)(K_{m2} + X_0)[V_{max1}(K_{m2} + X_0) + V_{max2}(K_{m1} + X_0)]}$$

The rate constant is defined as

$$\beta = V(X_0) X_0^{-h}$$

The analogous Generalized Mass Action representation, designated by the subscript GMA, is based on individual approximation of v_1 and v_2 and reads as follows:

$$V_{GMA} = \alpha_1 X^{g_1} + \alpha_2 X^{g_2}$$

where the kinetic orders g_1 and g_2 are defined as the logarithmic derivatives of v_1 and v_2 with respect to X at an operating point X_0:

$$g_1 = \left(\frac{\partial v_1}{\partial X}\right)\left(\frac{X}{v_1}\right) = \frac{K_{m1}}{K_{m1} + X_0}; \quad g_2 = \left(\frac{\partial v_2}{\partial X}\right)\left(\frac{X}{v_2}\right) = \frac{K_{m2}}{K_{m2} + X_0}$$

The rate constants are defined as follows:

$$\alpha_1 = v_1(X_0) X_0^{-g_1}; \quad \alpha_2 = v_2(X_0) X_0^{-g_2}$$

At first glance, V_S might seem to be a special case of V_{GMA} with $\beta = \alpha_1$, $h = g_1$ and $\alpha_2 = 0$, and one might be tempted to conclude that V_{GMA} must be the better representation since the parameters α_2 and g_2 could be adjusted to improve the representation. However, the parameters in V_{GMA} cannot be chosen freely; they are uniquely derived as logarithmic derivatives of v_1 and v_2 as shown above. Both V_S and V_{GMA} are computed from the same

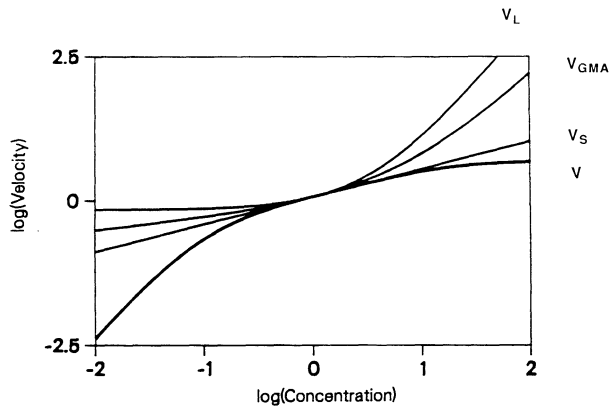


Figure 5. Accuracy of alternative representations of the diverging pathway of Fig. 3 (see Text for details).

rate laws v_1 and v_2, and the seemingly larger number of degrees of freedom in V_{GMA} does not help to improve the representation. In fact, V_{GMA} leads to an inferior representation. This is demonstrated best when V, V_S, and V_{GMA} are represented in logarithmic coordinates. There, all three representations have the operating point X_0 in common, V typically is concave downward, V_S is a straight line with slope h, and V_{GMA} is concave upward (Fig. 5). That is, at the operating point, V_S and V_{GMA} are exactly the same, but V_S is closer to V for all other concentrations and thus more accurate. Only if K_{m1} and K_{m2} differ by several orders of magnitude can mathematical examples be constructed where V_{GMA} yields a more accurate representation [cf. discussion of these rare cases in Voit & Savageau (1987)]. Both power-law representations, V_S and V_{GMA}, are superior to the linear approximation, V_L (Fig. 5).

Converging Pathways. The rate of product formation from a set of converging pathways (Fig. 4) is described within the framework of Michaelis-Menten kinetics as

$$V = v_1 + v_2 = \frac{V_{max1} X_1}{K_{m1} + X_1} + \frac{V_{max2} X_2}{K_{m2} + X_2}$$

This equation has an appearance similar to that for diverging pathways. However, two variables are involved now, which makes the comparison between *S*-system and Generalized Mass Action representations considerably more difficult. The two representations in this case are

$$V_S = \alpha X_1^{g_1} X_2^{g_2} \quad \text{and} \quad V_{GMA} = \beta_1 X_1^{h_1} + \beta_2 X_2^{h_2}$$

As in the previous case of diverging pathways, the kinetic orders in both representations are defined as relative derivatives with respect to X_1 and X_2. In the *S*-system representation, these derivatives are computed from the total rate V, whereas in the Generalized Mass Action representation they are derived from the individual rates v_1 and v_2.

The straightforward way of comparing accuracy of V_S and V_{GMA} would be to analyse the relative or absolute differences between V and V_S or V and V_{GMA} as functions of X_1 and X_2. However, the complex results of such analyses do not yield much insight, and a different approach is required.

Two methods seem appropriate (cf. Voit & Savageau, 1987). In one method, one studies the domain of points (X_1, X_2) for which the reference V and either of the power-law representations V_S or V_{GMA} differ by at most a given amount, for instance, 5% or 10%. Such comparisons demonstrate that V_S is virtually always more accurate than V_{GMA}. Fig. 6 shows an example for such a comparison when the error tolerance is 5%: the domain of accurate representation of V_{GMA} is completely contained within the domain of V_S; V_{GMA} is only more accurate than V_S for some irrelevant combinations of concentrations that cannot be shown with the resolution of Fig. 6. The superiority of V_S is qualitatively independent of the particular error tolerance selected and largely unaffected by the choice of K_{m1} and K_{m2}. Fig. 6 also shows the domain of validity for the linear representation V_L. This domain is contained within the domains of either power-law representation, and thus the linear representation is least accurate.

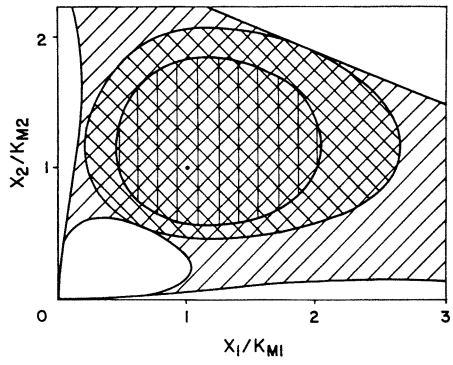


Figure 6. Accuracy of alternative representations of the converging pathway of Fig. 4 (see Text for details). The *S*-system representation (///) has the widest range of validity; the ranges of Generalized Mass Action (\\\) and linear (|||) representations are smaller. The error tolerance is 5%; other error tolerances produce qualitatively similar pictures.

Another type of comparison between alternative representations is based on the assumption that perturbations from the operating point occur in all directions with the same probability. One compares the average error for a given extent of perturbation and studies for which combinations of concentrations X_1 and X_2 either representation is more accurate. This type of analysis again shows that aggregation at the level of enzyme-catalysed reactions improves accuracy, since for all relevant concentrations the average error is smaller when one uses V_S rather than V_{GMA} (for details see Voit & Savageau, 1987). Again, both power-law representations are more accurate than the linear representation.

It is noted that the two methods of comparison are very different in character. The first one, similar to the analysis of diverging pathways, shows general superiority, at every relevant point. In particular, no randomness of perturbations is addressed; V_S is simply more accurate than V_{GMA} in the entire relevant domain. The second type of comparison is based on the statistical argument that the direction of a perturbation from the operating point is unpredictable and thus considered random. One could argue which of the two types of comparison is more appropriate. However, since V_S is superior in both cases, there is actually no need for such a discussion[1].

The result for both diverging and converging pathways is that the strategy of aggregation leading to the *S*-system representation improves accuracy over that leading to the Generalized Mass Action representation. This may be surprising since intuitively one might surmise that approximating the individual rates and subsequently summing their representations would be a better strategy then summing the individual rates and then approximating the result. However, power-law representations typically overestimate hyperbolically shaped functions and underestimate the summation function. The Generalized Mass Action representation uses the power-law representation only for the rates, whereas the *S*-system representation uses the power-law representation for both the rates and their

[1]The recent criticism of these comparisons in Cornish-Bowden (1989), in my opinion, is not justified. He has suggested that one should minimize maximum errors. This appears to be inappropriate since all three representations deviate from the Michaelis-Menten rate law by arbitrarily large amounts if one considers the complete range of substrate concentrations. Minimizing the maximum error under these conditions obviously is irrelevant, and one would again have to define relevant ranges over which the substrates were allowed to vary and in which one would minimize the maximum error.

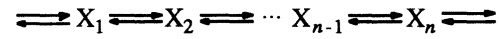


Figure 7. Simple amphibolic pathway.

summation. Thus, the latter approach is superior because it allows for cancellation of errors rather than their accumulation. This can be seen clearly when one examines accuracy in more complex integrated biochemical systems.

Accuracy of Representation for Integrated Pathways

In the previous section, comparisons involving the fundamental components of biochemical systems showed that the *S*-system representation is more accurate than the Generalized Mass Action representation, and on this basis one could argue that the *S*-system approach would be the superior strategy for any biochemical system. However, the validity of such a generalization is difficult to assess with mathematical rigour. As an alternative to mathematical proof, one can look at representative systems to gain experience with competing strategies for representation. Sorribas & Savageau (1989*abc*) have studied in detail two types of biochemical systems and compared representations based on different aggregation strategies. One type of system is an amphibolic pathway that raises the immediate question of whether and how one should aggregate fluxes, the other type of system involves enzyme-enzyme interactions. In the following, I shall review some of their results that are concerned with accuracy of representation.

Aggregation in Amphibolic Pathways. Some biochemical pathways cannot be considered essentially irreversible. As a matter of fact, the flux through some reactions changes direction under physiological conditions, and the question arises as to how accurately alternative aggregation strategies represent the behaviour of such pathways. Sorribas and Savageau (1989*c*) have investigated alternative strategies for representing amphibolic pathways of the type shown in Fig. 7.

The "irreversible" strategy involves aggregation of forward and reverse fluxes through each reaction (Fig. 8); this strategy has been selected in Metabolic Control Theory. The "reversible" strategy, in contrast, involves aggregation of incoming fluxes separately from outgoing fluxes for each metabolite (Fig. 9).

Sorribas and Savageau found that both strategies produce the same steady-state behaviour for the metabolite concentrations, and one could be tempted to disregard all further considerations if only the steady-state behaviour of the concentration variables is of interest. However, they found very different results for the behaviour of the flux in the system. Only the reversible strategy is capable of accurately representing the flux at or near thermodynamic equilibrium.

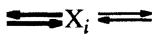


Figure 8. "Irreversible" strategy of aggregation. Two heavy arrows are aggregated into one term and two light arrows are aggregated into one term.

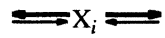


Figure 9. "Reversible" strategy of aggregation. Two heavy arrows are aggregated into one term and two light arrows are aggregated into one term.

Representations based on the irreversible strategy are extremely inaccurate and sensitive to changes in parameter values when the operating point is near equilibrium. Under these conditions one can obtain distorted results that deviate arbitrarily far from the correct results; the equilibrium point itself is a singularity and the system cannot be represented at this point. The implications of these results for the analysis of experimental data are clear; the response of a biochemical pathway to a realistic perturbation can be very different from the response to a theoretical, infinitesimal perturbation and the comparison between theory and experiment may be meaningless. Even when the operating point is far from equilibrium, the irreversible strategy leads to inferior representations, and the reversible strategy is always more accurate. These considerations unambiguously demonstrate that the accuracy of the reversible strategy is superior to that of the irreversible strategy in representing amphibolic pathways; other advantages are discussed at length in Sorribas & Savageau (1989*c*).

Enzyme-enzyme interactions. Sorribas & Savageau (1989*ab*) compared in detail several strategies for the representation of enzyme-enzyme interactions in a biochemical system. In particular, they compared and discussed various systemic representations and modes of analysis within Biochemical Systems Theory, Flux-Oriented Theory and Metabolic Control Theory.

Among their findings are comparative results on the accuracy of representation. The most important result may be that the Power-Law Formalism leads to impressively large ranges of variation within which the actual reference data and the power-law representations differ by less than a given error tolerance. For the particular biochemical system they investigated, the *S*-system representation of Biochemical Systems Theory yielded an average range of 20 fold variation within which the reference and power-law representations differed by less than 5%. The corresponding Generalized Mass Action representation within Biochemical Systems Theory produced an average range of about 15-fold. There is considerable evidence that these ranges are large enough to include variations typically observed in normal metabolic systems, and even many pathologic systems. For many metabolites the empirically determined ranges typically are measured in percent and only seldom exceed a few multiples of the norm [cf. collections of normal and pathological metabolic values, e.g. in Geigy (1960) or Wallach (1978)]. Thus, the ranges of valid power-law representation (e.g. with a maximum error of 5%) are sufficient to account for much of the relevant steady-state behaviour of biochemical systems. Sorribas & Savageau (1989*ab*) also compared the accuracy of representations based on *S*-systems or Generalized Mass Action systems with regard to their response to various perturbations. Their results support the conclusion that both power-law representations accurately capture the dynamic behaviour within the stated error tolerance, with the *S*-system representation outperforming the Generalized Mass Action representation by a small margin.

Conclusion

Biochemical Systems Theory as well as Metabolic Control Theory and Flux-Oriented Theory are based on logarithmic derivatives, which correspond directly or implicitly to the

Power-Law Formalism. In Biochemical Systems Theory, the power-law approach was selected with the explicitly stated intent to replace insufficiently accurate linear representations, while still allowing for analytical evaluations of steady-state characteristics. Representing the dynamics of biochemical systems also was of concern from the very beginning. For each of these objectives, the question of accuracy had to be addressed and this led to various analyses of the accuracy of power-law representations. In Metabolic Control Theory, questions of accuracy have not been an explicit issue, because only properties of a fixed steady-state have been targeted and all dynamical aspects have been excluded.

Considering that the ultimate goal is to understand metabolic networks *in vivo*, the restriction to steady-state characteristics is difficult to understand since such systems are constantly exposed to quite large "perturbations" that derive from the organism's changing metabolic demands, diet, disease, or drug treatment. Obviously, normal metabolism responds to these frequent changes in an organized fashion. Understanding pathologic as well as normal metabolic regulation seems to require consideration of large "perturbations" in external or independent variables and alterations in the structure of the metabolic network itself, not just infinitesimal changes.

In discussing the merits of alternative representations for biochemical networks, Biochemical Systems Theory has been considered by some to be problematic since the *S*-system representation is "only" an approximation that lacks exactness. Given that all mathematical representations of biochemical systems are approximations and that the exact mathematical forms are unknown, one should not dismiss approximations *per se*. Rather, one should select from alternative approximations the one that seems most appropriate according to objective criteria. Like any other approximation, the *S*-system representation is a compromise between the conflicting goals of generality, accuracy, and mathematical tractability.

The feature that distinguishes the *S*-system from the Generalized Mass Action representation is the strategy of aggregation used in generating the systemic descriptions. The *S*-system representation leads to deviations from mass balance at branch-points, which may be considered a disadvantage, but in my opinion this is more than compensated by a number of significant advantages that are realized with this strategy. In particular, the available evidence reviewed above indicates that the concentrations and fluxes in metabolic networks are more accurately represented with *S*-systems than with Generalized Mass Action systems. Even if one is only interested in steady-state aspects of biochemical systems, one must be concerned with accuracy of representation, since numerical results from a model are typically compared with experimentally measured responses that are not infinitesimal. In many cases, finite experimental perturbations may affect a metabolic system almost like an infinitesimal perturbation. However, if a system at a given operating point is sensitive to parameter variations, even very small perturbations can evoke responses that are significantly different from those corresponding to infinitesimal changes. This is most evident in the study of amphibolic pathways by Sorribas and Savageau (1989*c*), where it was demonstrated that an inappropriate representation can generate grossly distorted results in response to a very small perturbation.

In summary, the results presented here and elsewhere (Voit & Savageau, 1987; Sorribas & Savageau, 1989*abc*) show that the power-law representations that underlie Biochemical Systems Theory, Metabolic Control Theory and Flux-Oriented Theory accurately reflect Michaelis-Menten and Hill kinetics over wide variations in the substrate concentration. They are almost always more accurate than linear representations. Furthermore, the results suggest that the *S*-system variant of the Power-Law Formalism (Biochemical Systems Theory) typically produces more accurate representations than the Generalized Mass Action variants that underlie Flux-Oriented Theory and Metabolic Control Theory. Thus, the aggregation strategy that leads to the *S*-system representation is the strategy of choice when one considers the advantages of accuracy as well as the other advantages that have been discussed by Savageau in Chapter 4 of this book.

REFERENCES

Cornish-Bowden, A. (1989) *J. Theor. Biol. 136,* 365-377

Crabtree, B. & Newsholme, E. A. (1978) *Eur. J. Biochem. 89,* 19-22

Crabtree, B. & Newsholme, E. A. (1985) *Curr. Top. Cell. Reg. 52,* 21-76

Crabtree, B. & Newsholme, E. A. (1987) *Biochem. J. 247,* 113-120

Geigy, J. R. (1960) Documenta Geigy Wissenschaftliche Tabellen, J.R. Geigy S.A., Basle

Heinrich, R. & Rapoport, T. (1974) *Eur. J. Biochem., 42,* 89-95

Heinrich, R. & Rapoport, T. (1975) *BioSystems 7,* 130-136

Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104

Savageau, M. A. (1969*a*) *J. Theor. Biol. 25,* 365-369

Savageau, M. A. (1969*b*) *J. Theor. Biol. 25,* 370-379

Savageau, M. A. (1970) *J. Theor. Biol. 26,* 215-226

Savageau, M. A. (1971) *Arch. Biochem. Biophys. 145,* 612-621

Savageau, M. A. (1972) *Curr. Top. Cell Reg. 6,* 63-130

Savageau, M. A. (1975) *J. Mol. Evol. 5,* 199-222

Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*. Addison-Wesley, Reading, Massachusetts

Savageau, M. A. (1979) *Proc. Nat. Acad. Sci. 76,* 5413-5417

Savageau, M. A., Voit, E. O. & Irvine, D. H. (1987*a*) *Math. Biosci. 86,* 127-145

Savageau, M. A., Voit, E. O. & Irvine, D. H. (1987*b*) *Math. Biosci. 86,* 147-169

Sorribas, A. & Savageau, M. A. (1989*a*) *Math. Biosci. 94,* 161-193

Sorribas, A. & Savageau, M. A. (1989*b*) *Math. Biosci. 94,* 195-238

Sorribas, A. & Savageau, M. A. (1989*c*) *Math. Biosci. 94,* 239-269

Voit, E. O. & Savageau, M. A. (1987) *Biochemistry 26*, 6869-6880

Wallach, J. (1978) *Interpretation of Diagnostic Tests,* Little, Brown and Company, Boston

Chapter 6

Modern Control Theories: a Consumers' Test

ALBERT K. GROEN and HANS V. WESTERHOFF

DURING THE LAST decades several theoretical frameworks have been developed for a quantitative approach to studies of control of metabolism. Two important theories are *biochemical systems theory* developed by Savageau (1969) and *metabolic control analysis* formulated independently by Kacser & Burns (1973) and Heinrich & Rapoport (1974). Crabtree & Newsholme (1985, 1987) have combined aspects of these two theories in their treatment of control of metabolism. (This theory has been called *flux-oriented theory* by Savageau and colleagues, for example in Chapters 4 and 5 of this book, but no name is in general use). So far application of the theories to experimental practice has been relatively rare. To our knowledge, only control analysis has been applied to some extent by workers outside the group of the originators of the theories. This does not necessarily imply that control analysis is better than the other two theories. It does, however, suggest that control analysis is easier to understand for the mathematically untrained.

In this chapter a consumers' test of the three theories will be presented. We shall use the metabolic pathway of gluconeogenesis from lactate as test pathway. This pathway was chosen because detailed knowledge of intermediate concentrations at different gluconeogenic fluxes is present. Furthermore, the fluxes of the different segments of the pathway have been determined and the interaction of the pathway with other metabolic pathways in the cell has been established (Hue, 1981). The three theories will be compared on their ability to answer the following questions:

(1) Which steps exert significant control on pathway flux?
(2) What is the control by the enzymes on metabolite concentrations?
(3) What is the underlying mechanism for the control the enzymes exert?
(4) Which regulatory mechanism is most important for pathway control under physiological conditions?

Albert K. Groen ● Department of Gastroenterology, Academisch Medisch Centrum, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands ❖ Hans V. Westerhoff ● Division of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

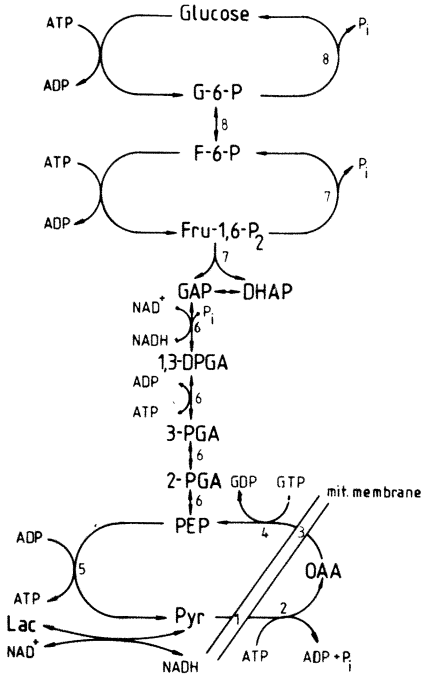


Figure 1. Scheme of gluconeogenesis

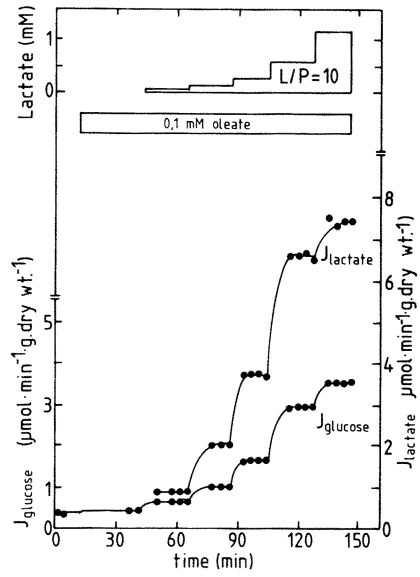


Figure 2. Titration of rat liver parenchymal cells with lactate and pyruvate in the presence of oleate. Rat liver cells (260 mg dry wt.) were perifused with different concentrations of lactate and pyruvate in the presence of 0.1 mM oleate as indicated in the figure. L/P, lactate/ pyruvate.

Experimental Conditions

The reactions involved in gluconeogenesis from lactate are given in Fig. 1. The gluconeogenic pathway contains five non-equilibrium steps under intracellular conditions, i.e. pyruvate carboxylase (reaction 2 in the scheme), transport of oxaloacetate from the mitochondria to the cytosol (reaction 3), phosphoenolpyruvate carboxykinase (reaction 4), fructose 1,6-bisphosphatase (reaction 7) and glucose-6-phosphatase (reaction 8). There are three substrate cycles that can be active: the phosphoenolpyruvate cycle, the fructose-6-phosphate/fructose-1,6-bisphosphate cycle and the glucose/glucose-6-phosphate cycle. The pathway is linked to other important metabolic pathways in the cell via the cytosolic and mitochondrial ATP/ADP ratio and the cytosolic and mitochondrial redox level. A prerequisite for a quantitative study of the control structure of a metabolic pathway is that the pathway can be studied under steady state conditions. We have studied control of gluconeogenesis under steady state conditions by incubating isolated rat hepatocytes in a perifusion system (Van der Meer & Tager, 1976). In such a system there is a continuous inflow and outflow of medium, which makes it possible to keep the concentrations of substrates and products constant. Furthermore, different steady states can be obtained with one preparation of cells and during the experiment aliquots of the cell suspension can be taken for the determination of intracellular metabolites.

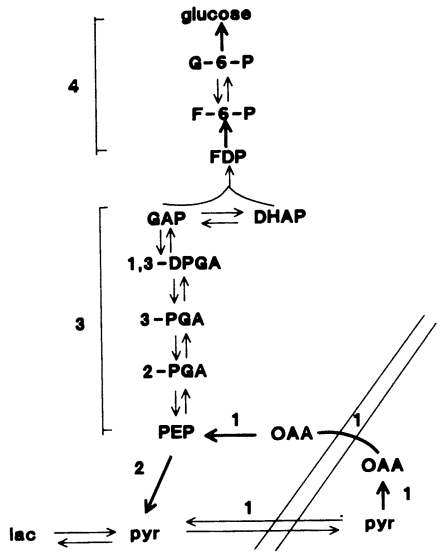


Figure 3. Simplified scheme of gluconeogenesis

A typical experiment is shown in Fig. 2. Cells were perifused with lactate and pyruvate in a constant concentration ratio of 10 in the presence of 0.1 mM oleate. Under these conditions the redox level is clamped. Upon increasing the lactate and pyruvate concentrations new steady-state rates of glucose synthesis ($J_{glucose}$) are obtained. Also given is the rate of lactate utilization ($J_{lactate}$). Since $2J_{lactate}$ is under all conditions almost equal to $J_{glucose}$, no carbon flows into other pathways. Under the conditions used there was no activity of the fructose-6-phosphate/fructose-1,6-bisphosphate cycle and the glucose/glucose-6-phosphate cycle. These cycles could therefore be ignored. To simplify the treatment further we have grouped reactions into the four important segments of the pathway. As shown in Fig. 3, the first segment is composed of the reactions between cytosolic pyruvate and phosphoenolpyruvate, the second segment is formed by pyruvate kinase. The third segment includes the reactions between phosphoenolpyruvate and glyceraldehyde-3-phosphate. The reactions between glyceraldehyde-3-phosphate and glucose constitute the fourth segment. We assumed that the triose phosphate isomerase reaction is in equilibrium.

Application of Metabolic Control Analysis

Metabolic control analysis is concerned with the relationships between *control coefficients* C that express the response of the whole system to a perturbation and *elasticity coefficients* ε that express the responses of individual enzymes to their substrates etc. The terminology and symbolism are derived from an agreement among several groups (Burns *et al.*, 1985), and as the basic ideas and definitions are fully set out in Chapter 3 of this book by Porteous, they will not be repeated here. Metabolic control analysis uses several basic equations from

which the control coefficients can be calculated, namely (i) the *summation theorems* for flux control and concentration control coefficients:

$$\sum_{i=1}^{n} C_{E_i}^{J} = 1; \quad \sum_{i=1}^{n} C_{E_i}^{S_j} = 0 \tag{1}$$

(ii) the *connectivity theorems* for flux control coefficients:

$$\sum_{i=1}^{n} C_{E_i}^{J} \cdot \varepsilon_{S_j}^{v_i} = 0 \tag{2}$$

and for concentration control coefficients:

$$\sum_{i=1}^{n} C_{E_i}^{S_k} \cdot \varepsilon_{S_j}^{v_i} = -\delta_{jk} \tag{3}$$

where $\delta_{jk} = 1$ if $j = k$ and 0 if $j \neq k$, and (iii) the *branching theorems* for flux control coefficients:

$$J_2 \cdot \sum_{\text{branch 1}} C_{E_i}^{J_4} + J_1 \cdot \sum_{\text{branch 2}} C_{E_i}^{J_4} = 0 \tag{4}$$

for concentration control coefficients for the pathway intermediates [not including initial substrate(s) and end product(s)]:

$$J_2 \cdot \sum_{\text{branch 1}} C_{E_i}^{S_k} + J_1 \cdot \sum_{\text{branch 2}} C_{E_i}^{S_k} = 0 \tag{5}$$

and for flux ratio control coefficients:

$$J_2 \cdot \sum_{\text{branch 1}} C_{E_i}^{J_2/J_4} + J_1 \cdot \sum_{\text{branch 2}} C_{E_i}^{J_2/J_4} = J_1 \tag{6}$$

For the gluconeogenic pathway simplified as shown in Fig. 3 the equations are summarized by the following matrix equation [Fell & Sauro, 1985, Sauro *et al.*, 1987, Westerhoff & Kell, 1987]:

$$\begin{bmatrix} C_1^{J_4} & -C_1^{PEP} & -C_1^{GAP} & C_1^{J_2/J_4} \\ C_2^{J_4} & -C_2^{PEP} & -C_2^{GAP} & C_2^{J_2/J_4} \\ C_3^{J_4} & -C_3^{PEP} & -C_3^{GAP} & C_3^{J_2/J_4} \\ C_4^{J_4} & -C_4^{PEP} & -C_4^{GAP} & C_4^{J_2/J_4} \end{bmatrix} = \begin{bmatrix} 1 & 1 & 1 & 1 \\ \varepsilon_{PEP}^{1} & \varepsilon_{PEP}^{2} & \varepsilon_{PEP}^{3} & 0 \\ 0 & 0 & \varepsilon_{GAP}^{3} & \varepsilon_{GAP}^{4} \\ J_2/J_1 & 1 & 0 & 0 \end{bmatrix}^{-1} \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} \tag{7}$$

In this and other equations, PEP and GAP represent phosphoenolpyruvate and glyceraldehyde-3-phosphate respectively.

When the elasticity coefficients and the flux through the different pathway segments can be determined, all control coefficients can be calculated. The gluconeogenic flux can be determined simply by measuring the amount of glucose produced. The pyruvate kinase flux J_2 was determined by addition of glucagon. This hormone fully inhibits pyruvate kinase, hence J_2 can be calculated from the glucagon-induced stimulation of gluconeogenic flux, J_4 (see Groen *et al.*, 1983 for a discussion). Of course, J_1 is equal to $J_2 + J_4$ (we here calculate J_4 in terms of C_3 equivalents). The elasticity coefficients of segment 2 and 4 to phosphoenolpyruvate and glyceraldehyde-3-phosphate respectively could be determined straightforwardly, since these segments are far from equilibrium and not inhibited by product. Therefore these elasticity coefficients can be calculated from simple plots of rate against substrate concentration. The slopes of the double logarithmic plots in Fig. 4 give the values for the elasticity coefficients directly. Note that these plots are linear for a wide range of metabolite concentrations. The reactions in segment 3 are close to equilibrium. Therefore the elasticity coefficients of segment 3 to phosphoenolpyruvate and glyceraldehyde-3-phosphate could be calculated from the ratio between the combined mass-action ratio and the combined equilibrium constant for these reactions (Γ/K_{eq}) using the following equations (see Groen *et al.*, 1982)

$$\varepsilon^3_{PEP} = \frac{1}{1 - \Gamma/K_{eq}} \tag{8}$$

and

$$\varepsilon^3_{GAP} = \frac{-\Gamma/K_{eq}}{1 - \Gamma/K_{eq}} \tag{9}$$

The elasticity coefficient of segment 1 towards phosphoenolpyruvate was more difficult to determine. Since the magnitude of this elasticity coefficient did not significantly effect the calculated control distribution we have assumed it to be equivalent to the elasticity coefficient to pyruvate carboxylase with respect to mitochondrial oxaloacetate. This will overestimate the value of the actual elasticity coefficient somewhat. The values for the elasticity coefficients and fluxes through the pathway segments are listed in Table 1.

By inverting the matrix containing the values of the elasticity coefficients and the flux ratios, the control distribution in the gluconeogenic pathway as given by control analysis is obtained. Hence the control distribution is as follows:

Table 1. Values for elasticity coefficients of pathway segments and the pathway fluxes. Cells were perifused as described in the legend to Fig. 2. The elasticity coefficients and fluxes are given for the condition with saturating concentrations of lactate and pyruvate. It should be noted that J_4 is counted in terms of C_3 equivalents.

Parameter	Pathway segment			
	1	2	3	4
ε_{PEP}	-0.04	3.5	2.04	0
ε_{GAP}	0	0	-1.05	1.20
J	15.3	4.5	10.8	10.8

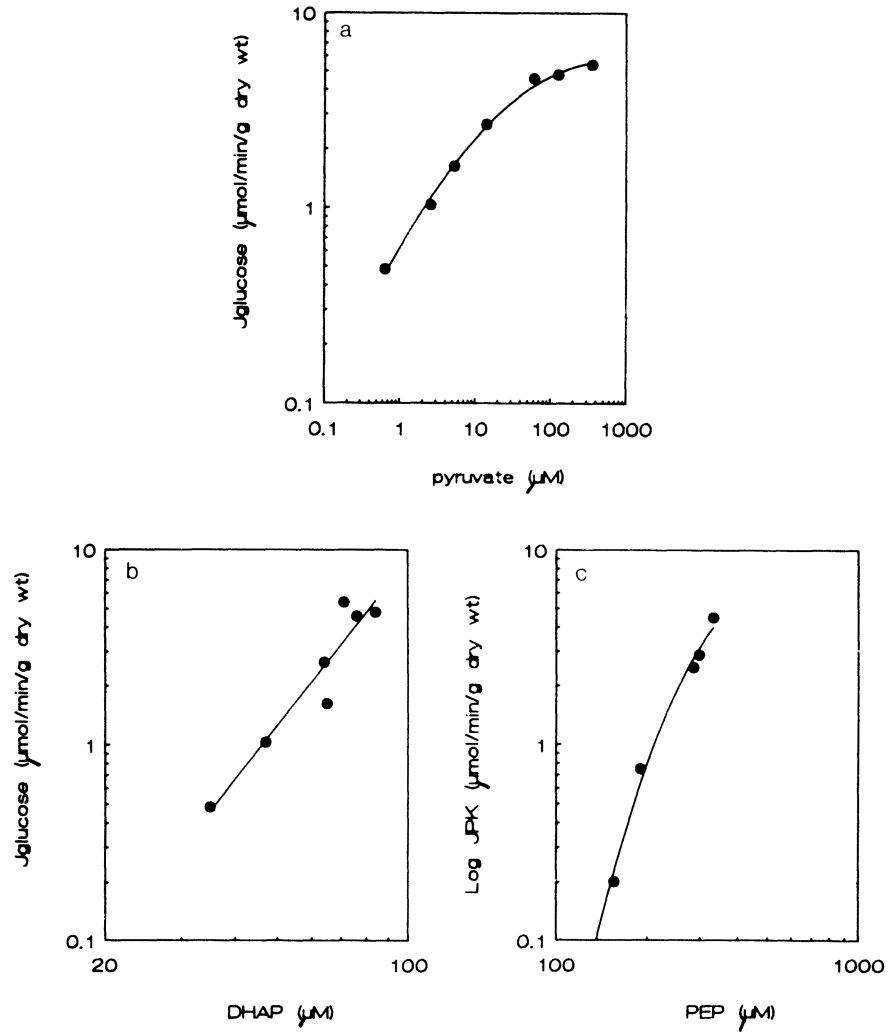


Figure 4. Relationship between the rate of glucose formation and the concentration of gluconeogenic intermediates. Rat-liver cells (280-300 mg dry weight) from starved rats were perifused with different concentrations of lactate and pyruvate in the presence of 0.1 mM oleate. In each steady state of glucose formation a sample of the cell suspension was taken for the determination of intracellular phosphoenolpyruvate and dihydroxyacetone phosphate. Pyruvate was assayed in the perifusate.

$$
\begin{array}{l}
\\
\text{Segment 1} \\
\text{Segment 2} \\
\text{Segment 3} \\
\text{Segment 4}
\end{array}
\begin{array}{c}
\begin{array}{cccc} C^{J_4} & C^{\mathrm{PEP}} & C^{\mathrm{GAP}} & C^{J_2/J_4} \end{array} \\
\begin{bmatrix}
0.59 & 0.54 & 0.49 & 1.31 \\
-0.17 & -0.16 & -0.15 & 0.62 \\
0.31 & -0.20 & 0.26 & -1.03 \\
0.27 & -0.18 & -0.61 & -0.90
\end{bmatrix}
\end{array}
\tag{10}
$$

Control of gluconeogenic flux (J_4) is distributed among the different pathway segments. Pyruvate kinase (segment 2) of course exerts negative control both on gluconeogenic flux and on the concentrations of phosphoenolpyruvate and glyceraldehyde-3-phosphate. Surprisingly, the near-equilibrium enzymes between phosphoenolpyruvate and glyceraldehyde-3-phosphate also exert flux control, even more so than the non-equilibrium enzymes in segment 4. This is due to the higher concentration control coefficient of segment 3 to phosphoenolpyruvate in combination with a very high elasticity coefficient of pyruvate kinase to phosphoenolpyruvate.

The control coefficients in the final column of the matrix of control coefficients found by this method are of interest because they relate to the extent of substrate cycling. Not surprisingly, an increased activity of pyruvate kinase and a decreased activity of segments 3 and 4 enhance cycling relative to gluconeogenic flux. *A priori* it was not as clear that an increased activity of segment 1 enhances cycling more than gluconeogenesis. Clearly at these high concentrations of pyruvate, pyruvate kinase functions as an overflow control system.

We have also calculated the control distribution in the gluconeogenic pathway at lower concentrations of lactate and pyruvate. In Fig. 5 the flux control coefficients of the four segments on gluconeogenic flux is plotted as a function of the flux. The flux was varied by titrating lactate and pyruvate keeping their ratio constant (Fig. 2). There is gradual shift of flux control to the first segment as flux decreases. It turns out that this is due to the decrease in pyruvate kinase flux at the lower lactate and pyruvate concentrations. Pyruvate kinase also influences the distribution of concentration control to a considerable extent (Fig. 6). The first pathway segment has a relatively high concentration control coefficient at all fluxes. Pyruvate kinase has a low concentration control coefficient on both intermediates because of its very high elasticity coefficient to phosphoenolpyruvate. This very high elasticity coefficient leads to a buffering of the concentrations of phosphoenolpyruvate and glyceraldehyde-3-phosphate. This is best demonstrated by the concentration control coefficient of segment 4

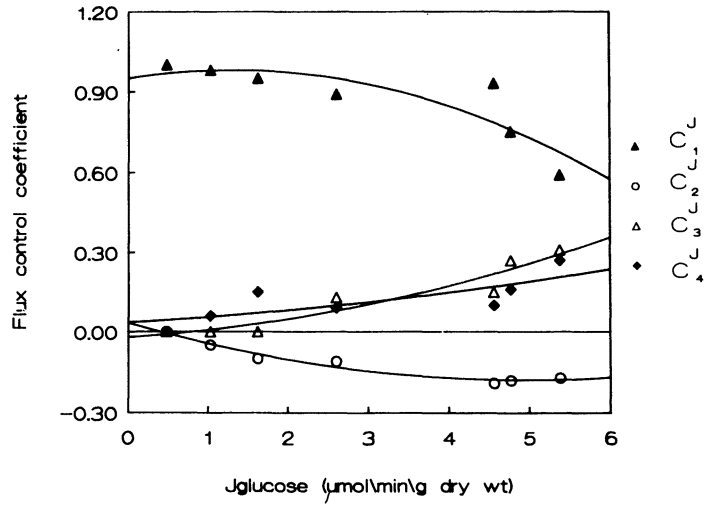


Figure 5. The flux control coefficients of the four segments of the gluconeogenic pathway as a function of gluconeogenic flux. For experimental details see the legend to Fig. 4. The flux control coefficients were calculated as described in the text.

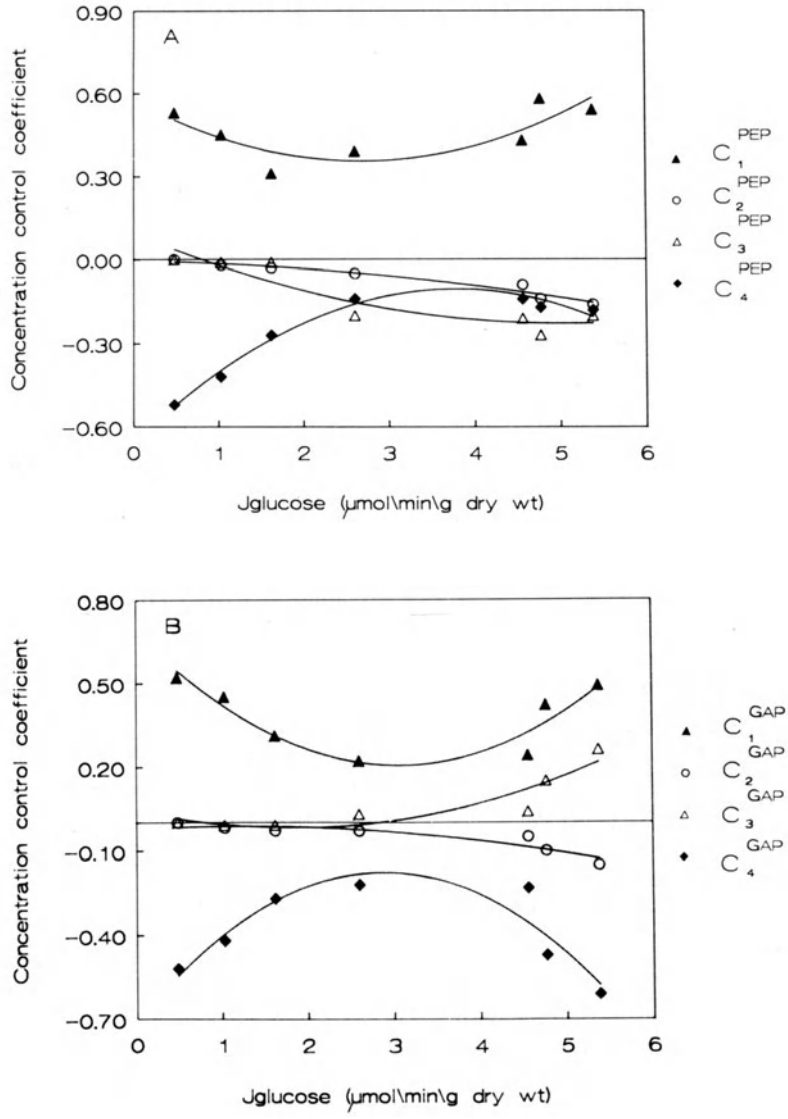


Figure 6. Relationship between gluconeogenic flux and the concentration control coefficients of four gluconeogenic pathway segments on the concentration of phosphoenolpyruvate (A) and dihydroxyacetone phosphate (B). For experimental details see the legend to Fig. 4. The concentration control coefficients were calculated as described in the text.

on phosphoenolpyruvate. At low gluconeogenic flux pyruvate kinase flux is almost zero (Groen *et al.*, 1983); because of the allosteric kinetics of pyruvate kinase to phosphoenolpyruvate the elasticity coefficient of the enzyme to this substrate is low. Then the concentration control coefficient of segment 4 on phosphoenolpyruvate is relatively high. At increasing flux, hence increasing the elasticity coefficient of pyruvate kinase to phosphoenol-

pyruvate, the concentration control coefficient of segment 4 on phosphoenolpyruvate decreases, showing the buffering effect of pyruvate kinase on the concentration control coefficient of phosphoenolpyruvate.

In the calculation presented above we have used the summation theorems. This implies that we have assumed that all reactions involved in the conversion of lactate have been included. The question arises whether this assumption is correct. Although we have clamped the redox level it was not possible to keep the ATP/ADP ratio in the cells constant. Therefore ATP producing and utilizing reactions could exert control on gluconeogenesis. Indeed we have shown that gluconeogenic flux is controlled to some extent by the adenine nucleotide translocator (Groen *et al.*, 1986). To obtain the real values of the control coefficients some of them, i.e. one for each branch point, have to be determined by direct experimental measurement. For the gluconeogenic pathway it turns out that the amount of negative "external" control almost compensates the amount of positive "external" control so that the actual values of the control coefficients do not change to a great extent (Groen *et al.*, 1986). Summarizing, by using control analysis flux control coefficients and concentration control coefficients can be calculated in a relatively simple way. Although application of the matrix method has greatly simplified the actual calculation of the control coefficients, the deeper understanding in the mechanisms underlying control by enzymes provided by inspection of the separate connectivity relations is lost when this method is used. Calculation of control coefficients does not reveal directly which step is most important for dynamic pathway control under physiological conditions. For instance, pyruvate kinase exerts only little control on flux and concentrations of metabolites. Yet it plays a central role in control of gluconeogenesis. This role becomes clear only after careful scrutiny of the relations between elasticity, concentration control and flux control coefficients.

The Matrix Method of Heinrich and Rapoport

A matrix method for calculation of concentration control coefficients was already published by Heinrich and Rapoport in 1975. They have applied this method to glycolysis in erythrocytes (Rapoport *et al.*, 1976). However, presumably because they have failed to explain in detail how the calculation procedures should be carried out the method has not been applied by others.

This method focusses on the net production rates of metabolites rather than on net reaction rates (fluxes). These are denoted as f_i, i.e., the net production flux of metabolite *i*. At steady state all f_i values are zero. After transitions between steady states, as caused by parameter changes, the change in f_i must of course also be zero. This can be expressed as follows:

$$\sum_j \eta_j^i \cdot C_r^{S_j} + \phi_r^i = 0 \tag{11}$$

where

$$\eta_j^i = \frac{df_i}{d\ln[S_j]} \tag{12}$$

and

$$\phi_j^i = \frac{df_i}{d\ln[E_j]} = \frac{df_i}{d\ln[v_j]} = v_i \frac{df_i}{dv_j} \tag{13}$$

Because f_i is zero the regular elasticity coefficients in which the logarithm of f_i is taken could not be used in these equations. Note that ϕ and η are analogues of the absolute elasticity coefficients of Westerhoff & van Dam (1987).

Rewriting eqn. (11) in matrix form one obtains:

$$[C] = -[\eta]^{-1}[\phi] \tag{14}$$

Here [C] is the $m \times n$ matrix of concentration control coefficients (with respect to enzyme changes), $[\eta]$ is the $m \times m$ matrix of η coefficients and $[\phi]$ is the $m \times n$ matrix of ϕ coefficients, where m and n are the number of freely variable metabolites and the number of enzymes respectively.

For the gluconeogenic pathway given in Fig. 3, f_{PEP} and f_{GAP} correspond to the net rates of synthesis of phosphoenolpyruvate and glyceraldehyde-3-phosphate respectively:

$$f_{GAP} = v_3 - v_4 \tag{15}$$

$$f_{PEP} = v_1 - v_2 - v_3 \tag{16}$$

We denote the change of f_{PEP} with the logarithm of the activity of enzyme 1 by ϕ_1^{PEP} [cf. eqn. (13)]. Using eqns. (13) and (16), we find for this coefficient:

$$\phi_1^{PEP} = v_1 \frac{df_{PEP}}{dv_1} = v_1$$

and analogously, for the whole set of ϕ values:

$$[\phi] = \begin{bmatrix} \phi_1^{PEP} & \phi_2^{PEP} & \phi_3^{PEP} & \phi_4^{PEP} \\ \phi_1^{GAP} & \phi_2^{GAP} & \phi_3^{GAP} & \phi_4^{GAP} \end{bmatrix} = \begin{bmatrix} v_1 & -v_2 & -v_3 & 0 \\ 0 & 0 & v_3 & -v_4 \end{bmatrix} = \begin{bmatrix} 15.3 & -4.5 & -10.8 & 0 \\ 0 & 0 & 10.8 & -10.8 \end{bmatrix} \tag{17}$$

Similarly we denote the change of f_{PEP} with the concentration of phosphoenolpyruvate by η_{PEP}^{PEP}, as defined by eqn. (12). For this coefficient we find:

$$\eta_{PEP}^{PEP} = \frac{dv_1}{d\ln[PEP]} - \frac{dv_2}{d\ln[PEP]} - \frac{dv_3}{d\ln[PEP]} = v_1\varepsilon_{PEP}^1 - v_2\varepsilon_{PEP}^2 - v_3\varepsilon_{PEP}^3$$

and similarly, for the other coefficients:

$$[\eta] = \begin{bmatrix} \eta_{PEP}^{PEP} & \eta_{GAP}^{PEP} \\ \eta_{GAP}^{GAP} & \eta_{GAP}^{GAP} \end{bmatrix} = \begin{bmatrix} v_1\varepsilon_{PEP}^1 - v_2\varepsilon_{PEP}^2 - v_3\varepsilon_{PEP}^3 & -v_3\varepsilon_{GAP}^3 \\ v_3\varepsilon_{PEP}^3 & v_3\varepsilon_{GAP}^3 - v_4\varepsilon_{GAP}^4 \end{bmatrix} = \begin{bmatrix} -38.39 & 11.34 \\ 22.03 & -24.3 \end{bmatrix} \tag{18}$$

Written in matrix form, eqn. (11) reads for this case:

$$\begin{bmatrix} \eta_{PEP}^{PEP} & \eta_{GAP}^{PEP} \\ \eta_{GAP}^{GAP} & \eta_{GAP}^{GAP} \end{bmatrix} \cdot \begin{bmatrix} C_1^{PEP} & C_2^{PEP} & C_3^{PEP} & C_4^{PEP} \\ C_1^{GAP} & C_2^{GAP} & C_3^{GAP} & C_4^{GAP} \end{bmatrix} + \begin{bmatrix} \phi_1^{PEP} & \phi_2^{PEP} & \phi_3^{PEP} & \phi_4^{PEP} \\ \phi_1^{GAP} & \phi_2^{GAP} & \phi_3^{GAP} & \phi_4^{GAP} \end{bmatrix} = \begin{bmatrix} 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix} \tag{19}$$

The meaning of this equation can be understood by considering the upper left element of the resulting matrix. This element represents the effect of a change in the activity of enzyme 1 on the production rate of phosphoenolpyruvate (f_{PEP}). Because in transitions between steady states, there cannot be a change in net production rate of any metabolite, this effect must be nil. The effect consists of the increase in f_{PEP} caused by changes in the phosphoenolpyruvate and glyceraldehyde-3-phosphate concentrations (two terms arising in the product of the two matrices on the left) and the increase in f_{PEP} due to the direct effect of the increase in enzyme 1 through the increase in v_1. As indicated by eqn. (14), eqn. (19) can be solved for the matrix of control coefficients:

$$[C] = \begin{bmatrix} C_1^{PEP} & C_2^{PEP} & C_3^{PEP} & C_4^{PEP} \\ C_1^{GAP} & C_2^{GAP} & C_3^{GAP} & C_4^{GAP} \end{bmatrix} = \begin{bmatrix} 0.54 & -0.16 & -0.20 & -0.18 \\ 0.49 & -0.15 & 0.26 & -0.61 \end{bmatrix} \tag{20}$$

The flux control coefficients can be calculated from the concentration control coefficients by using the relation of Heinrich *et al.* (1977) that connects the control coefficients to the elasticity coefficients:

$$C_r^{v_i} = \sum_k \varepsilon_k^i \cdot C_r^k + \delta_{ir} \tag{21}$$

where $\delta_{ir} = 1$ if $i = r$ and otherwise 0. For instance for the control exerted by enzyme 1 on the flux through segment 4 we find:

$$C_1^{J_4} = C_1^{GAP} \cdot \varepsilon_{GAP}^4 = 0.59 \tag{22}$$

Compared with the matrix method of the previous section, we find that this one has the advantage that the matrix that has to be inverted is smaller, i.e., $m \times m$, where m is the number of metabolites, rather than $n \times n$,where n is the number of enzymes. Especially with highly branched pathways and if matrix inversion has to be done without computer assistance, this may be an advantage. A disadvantage of this method is that after matrix inversion only the concentration control coefficients are known. For the flux control coefficients additional calculations have to be made. The method provides little information about the mechanism by means of which the enzymes exert control.

Application of the Control Theory of Crabtree & Newsholme

The basic equations in the control theory (*flux-oriented theory* in the terminology of Savageau and Voit in Chapters 4 and 5 respectively) of Crabtree & Newsholme (1987) are as follows:

$$d\ln J_i = d\ln E_i + \sum_{j=1}^{n} \varepsilon_{S_j}^{v_i} \cdot d\ln S_j \tag{23}$$

$$\tau_i \cdot J_i \cdot d\ln J_i = \sum_{j=1}^{n} \tau_j \cdot J_j \cdot d\ln J_j \tag{24}$$

where τ is the stoicheiometry between the fluxes. To avoid confusion we have used the nomenclature proposed by Burns *et al.* (1985).

Eqn. (23) corresponds to eqn. (21) multiplied by $d\ln E_i$. This is the equation relating flux control coefficients to concentration control coefficients and elasticity coefficients developed by Heinrich *et al.* (1977).

When applied to the gluconeogenic pathway given in Fig. 3 and written in matrix notation the equation is as follows:

$$\begin{bmatrix} d\ln E_1 \\ d\ln E_2 \\ d\ln E_3 \\ d\ln E_4 \\ 0 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 & -\varepsilon^1_{PEP} & 0 \\ 0 & 1 & 0 & -\varepsilon^2_{PEP} & 0 \\ 0 & 0 & 1 & -\varepsilon^3_{PEP} & -\varepsilon^3_{GAP} \\ 0 & 0 & 1 & 0 & -\varepsilon^4_{GAP} \\ -J_1 & J_2 & J_4 & 0 & 0 \end{bmatrix} \begin{bmatrix} d\ln J_1 \\ d\ln J_2 \\ d\ln J_3 \\ d\ln[PEP] \\ d\ln[GAP] \end{bmatrix} \tag{25}$$

The above equation can be understood by doing the following thought experiment: Activate E_1 by a certain fraction. This will cause a change in the flux through the enzyme by $d\ln E_1$ minus a secondary effect on this flux caused by changes in metabolites that affect v_1. The latter change equals $\varepsilon^1_{PEP} d\ln[PEP]$. This consideration gives the first row of eqn. (25). Similar considerations give the next three rows. The bottom row reflects the fact that the sum of the change in fluxes at the branch point must equal 0.

Inversion of the matrix of coefficients generates directly the values for all control coefficients in the pathway. Compared to control analysis this method is even more straightforward. In addition it has the advantage that the control coefficients with respect to all pathway fluxes are calculated in a single procedure. When applied to gluconeogenesis at saturating concentrations of lactate and pyruvate (see Fig. 3) the following control matrix is obtained:

$$\begin{bmatrix} C^{J_1}_{E_1} & C^{J_1}_{E_2} & C^{J_1}_{E_3} & C^{J_1}_{E_4} \\ C^{J_2}_{E_1} & C^{J_2}_{E_2} & C^{J_2}_{E_3} & C^{J_2}_{E_4} \\ C^{J_3}_{E_1} & C^{J_3}_{E_2} & C^{J_3}_{E_3} & C^{J_3}_{E_4} \\ C^{PEP}_{E_1} & C^{PEP}_{E_2} & C^{PEP}_{E_3} & C^{PEP}_{E_4} \\ C^{GAP}_{E_1} & C^{GAP}_{E_2} & C^{GAP}_{E_3} & C^{GAP}_{E_4} \end{bmatrix} = \begin{bmatrix} 0.98 & 0.01 & 0.01 & 0.01 \\ 1.90 & 0.44 & -0.72 & -0.63 \\ 0.59 & -0.17 & 0.31 & 0.27 \\ 0.54 & -0.16 & -0.20 & -0.18 \\ 0.49 & -0.15 & 0.26 & -0.61 \end{bmatrix} \tag{26}$$

The values for the flux control coefficients on gluconeogenic flux and the concentration control coefficients are identical to those calculated by control analysis. The differences in the control structure within the different segments of the pathway are nicely uncovered in

this control matrix. Clearly the first segment is an uncontrolled pathway whereas control is distributed in the other pathway segments. An essential prerequisite when applying this method is that elasticity coefficients of all the effectors of enzyme activity have to be included in the equations. Crabtree & Newsholme (1987) have chosen to differentiate between different types of enzyme regulators. Again we have simplified the treatment by ignoring the possible influence of, for instance, the ATP/ADP ratio. The consequences of such a simplification for the control distribution are not as transparent as in the case of control analysis. This is due to the lack of the connectivity theorems. It could be that the lack of information about mechanisms for control generated by the treatment of Crabtree & Newsholme (1985, 1987) has led them to introduce a new class of very confusing qualitative terms such as *totally external* or *partially internal* regulators.

Summarizing, by using the control theory of Crabtree & Newsholme (1987) control coefficients can be calculated in an extremely simple way. The method has the advantage that control coefficients on all fluxes are obtained simultaneously. It has the disadvantage that very little information is obtained about the mechanism by which control is exerted.

Application of Biochemical Systems Theory

As discussed in detail by Savageau and Voit in Chapters 4 and 5 respectively of this book, there are several ways in which biochemical systems theory can be used to describe biochemical systems. In one, the *generalized mass-action* system, the rate of every enzyme catalysed reaction is approximated by a difference between power laws. In another, the *S-system* representation, the net production rate of each metabolite [f_i in eqns. (12) and (27)] is approximated by such a difference. The reactions producing and the reactions consuming that metabolite are then represented by single power laws. Using the latter approach, the basic equation is as follows (Savageau, 1976):

$$f_i = \frac{dX_i}{dt} = \alpha_i \prod_{j=1}^{n+m} X_j^{g_{ij}} - \beta_i \prod_{j=1}^{n+m} X_j^{h_{ij}} \tag{27}$$

$$\alpha_i = v_{i0} \prod_{j=1}^{n} X_{j0}^{-g_{ij}} \tag{28}$$

$$\beta_i = v_{i0} \prod_{j=1}^{n} X_{j0}^{-h_{ij}} \tag{29}$$

where subscript 0 refers to any reference state. In case X_i is not at a branch point, the first term on the right hand side of eqn. (27) can be interpreted as an approximation to the net rate of the reaction producing X_i, the second term approximating the net rate of the reaction consuming X_i. In this interpretation g_{ij} corresponds to the elasticity coefficient of the former reaction with respect to metabolite X_j, whereas h_{ij} is equal to the elasticity coefficient of the consuming reaction with respect to any metabolite X_j. If X_i is at a branch point in the pathway, as is phosphoenolpyruvate in our case, either or both terms on the right

hand side of eqn. (27) describe the sum of the rates of two (or more) enzyme catalysed reactions. Then the g and h values can no longer be identified as elasticity coefficients in the usual sense. They are closer to the "overall elasticity coefficients" (Westerhoff *et al.*, 1984) describing the combined elasticity of a part of a metabolic pathway. Since one is often more interested in the effect of the molecular elasticity coefficients on the control distribution, this is a disadvantage. However, it is still possible to express the g and h values as elasticity coefficients and enzyme parameters. We shall illustrate this by elaborating our pathway in somewhat more detail. The biochemical systems theory (S-system) equations for the gluconeogenic pathway in Fig. 3 are as follows:

$$f_{PEP} = \frac{d[PEP]}{dt} = \alpha_{PEP} \cdot [PEP]^{g_{PEP,PEP}} \cdot [GAP]^{g_{PEP,GAP}} - \beta_{PEP} \cdot [PEP]^{h_{PEP,PEP}} \cdot [GAP]^{h_{PEP,GAP}} \quad (30)$$

$$f_{GAP} = \frac{d[GAP]}{dt} = \alpha_{GAP} \cdot [PEP]^{g_{GAP,PEP}} \cdot [GAP]^{g_{GAP,GAP}} - \beta_{GAP} \cdot [PEP]^{h_{GAP,PEP}} \cdot [GAP]^{h_{GAP,GAP}} \quad (31)$$

Note that in biochemical systems theory α and g are used for the rate constant and exponents respectively of the synthetic reactions, whereas β and h are used as rate constants and exponents respectively in the degradative reactions.

The corresponding integral description in the generalization of metabolic control theory is as follows:

$$v_1 = k_1 [PEP]^{\varepsilon^1_{PEP}} \quad (32)$$

$$v_2 = k_2 \cdot [PEP]^{\varepsilon^2_{PEP}} \quad (33)$$

$$v_3 = k_3 \cdot [PEP]^{\varepsilon^3_{PEP}} \cdot [GAP]^{\varepsilon^3_{GAP}} \quad (34)$$

$$v_4 = k_4 \cdot [GAP]^{\varepsilon^4_{GAP}} \quad (35)$$

This is the integral equivalent of the metabolic control theory description, which, like biochemical systems theory, is strictly valid in the limit of infinitesimal changes around an operating point. Note that we use k_j for the rate constant of the reaction catalysed by a single enzyme j. The first term on the right-hand side of eqn. (30) is identical to v_1, the first term on the right-hand side of eqn. (31) is v_3 and the second term on the right-hand side of eqn. (31) is v_4. Thus α_{PEP}, α_{GAP} and β_{GAP} are proportional to the activities of enzyme 1, segment 3 and segment 4 respectively. Furthermore, we identify:

$$\begin{aligned} g_{PEP,PEP} = \varepsilon^1_{PEP};\quad g_{PEP,GAP} = 0;\quad g_{GAP,PEP} = \varepsilon^3_{PEP};\quad g_{GAP,GAP} = \varepsilon^3_{GAP};\\ h_{GAP,PEP} = 0;\quad h_{GAP,GAP} = \varepsilon^4_{GAP} \end{aligned} \quad (36)$$

The second term on the right hand side of eqn. 30 constitutes more of an identification problem, because it represents a sum of reaction rates, i.e. of v_2 and v_3.

The correspondence relations between the two descriptions are found by requiring that in the standard state the two descriptions are equal both (i) with respect to the predicted rate and (ii) with respect to the predicted dependence of the rate on the concentrations of the metabolites. Condition (i) gives the following relationship (where the concentrations are those in the reference state):

$$\beta_{PEP} = \frac{k_2 \cdot [PEP]^{\varepsilon^2_{PEP}} + k_3 \cdot [PEP]^{\varepsilon^3_{PEP}} \cdot [GAP]^{\varepsilon^3_{GAP}}}{[PEP]^{h_{PEP,PEP}} \cdot [GAP]^{h_{PEP,GAP}}} \tag{37}$$

Condition (ii) implies two more equations for the derivatives of the logarithms of this equation with respect to the concentrations of phosphoenolpyruvate and glyceraldehyde-3-phosphate respectively, again at the reference state:

$$h_{PEP,PEP} = \frac{k_2 \cdot \varepsilon^2_{PEP} \cdot [PEP]^{\varepsilon^2_{PEP}} + k_3 \cdot \varepsilon^3_{PEP} \cdot [PEP]^{\varepsilon^3_{PEP}} \cdot [GAP]^{\varepsilon^3_{GAP}}}{v_1} = \frac{v_2 \cdot \varepsilon^2_{PEP} + v_3 \cdot \varepsilon^3_{PEP}}{v_1} = 2.47 \tag{38}$$

$$h_{PEP,GAP} = \frac{k_3 \cdot [PEP]^{\varepsilon^3_{PEP}} \cdot \varepsilon^3_{GAP} \cdot [GAP]^{\varepsilon^3_{GAP}}}{v_1} = v_3 \cdot \varepsilon^3_{GAP} / v_1 = -0.74 \tag{39}$$

In the steady state eqns. (30) and (31) are both equal to zero. Division by their right-hand terms and taking logarithms, leads to the following, written as a matrix equation:

$$\begin{bmatrix} \ln[PEP] \\ \ln[GAP] \end{bmatrix} = [A]^{-1} \begin{bmatrix} \ln(\alpha_{PEP}/\beta_{PEP}) \\ \ln(\alpha_{GAP}/\beta_{GAP}) \end{bmatrix} \tag{40}$$

with

$$[A] = \begin{bmatrix} g_{PEP,PEP} - h_{PEP,PEP} & g_{PEP,GAP} - h_{PEP,GAP} \\ g_{GAP,PEP} - h_{GAP,PEP} & g_{GAP,GAP} - h_{GAP,GAP} \end{bmatrix} = \begin{bmatrix} -2.51 & 0.741 \\ 2.04 & -2.25 \end{bmatrix} \tag{41}$$

The elements of the inverse matrix, which we shall call $[A]^{-1}$, give the control coefficients corresponding to the control exerted by the α and β values on the concentrations of phosphoenolpyruvate and glyceraldehyde-3-phosphate. However, these control coefficients do not directly correspond to the control coefficients with respect to the various enzyme activities. We can find these latter ones by realizing that:

$$C_i^{PEP} = \frac{d\ln[PEP]}{d\ln E_i} = \frac{d\ln[PEP]}{d\ln(\alpha_{PEP}/\beta_{PEP})} \cdot \frac{d\ln(\alpha_{PEP}/\beta_{PEP})}{d\ln E_i} + \frac{d\ln[PEP]}{d\ln(\alpha_{GAP}/\beta_{GAP})} \cdot \frac{d\ln(\alpha_{GAP}/\beta_{GAP})}{d\ln E_i} \tag{42}$$

The $\partial\ln(\alpha/\beta)/\partial\ln E_i$ values can be evaluated from the correspondences discussed above between the α and β values and the enzyme activities. The dependence of $\ln(\alpha_{GAP}/\beta_{GAP})$ is the simplest: 0, 0, 1, and -1 for enzymes 1, 2, 3, and 4, respectively. The dependence of $\ln(\alpha_{PEP}/\beta_{PEP})$ on enzymes 1 and 4 is also simple: 1 and 0, respectively. However, its dependence on the other two enzymes is more complex, as follows, from eqn. (37):

$$\frac{d\ln(\alpha_{PEP}/\beta_{PEP})}{d\ln E_2} = \frac{-\alpha_2 \cdot [PEP]^{\varepsilon^2_{PEP}}}{\alpha_2 \cdot [PEP]^{\varepsilon^2_{PEP}} + \alpha_3 \cdot [PEP]^{\varepsilon^3_{PEP}} \cdot [GAP]^{\varepsilon^3_{GAP}}} = -v_2/(v_2 + v_3) \tag{43}$$

and

$$\frac{d\ln(\alpha_{PEP}/\beta_{PEP})}{d\ln E_3} = \frac{-\alpha_3 \cdot [PEP]^{\varepsilon^3_{PEP}} \cdot [GAP]^{\varepsilon^3_{GAP}}}{\alpha_2 \cdot [PEP]^{\varepsilon^2_{PEP}} + \alpha_3 \cdot [PEP]^{\varepsilon^3_{PEP}} \cdot [GAP]^{\varepsilon^3_{GAP}}} = -v_3/(v_2 + v_3) \tag{44}$$

The total result can be formulated as follows:

$$[C] = -[A]^{-1}[D] \tag{45}$$

where [D] contains all these terms connecting the $\partial\ln(\alpha/\beta)$ values to the $\partial\ln E_i$ values:

$$[D] = \begin{bmatrix} 1 & -v_2/v_1 & -v_3/v_1 & 0 \\ 0 & 0 & 1 & -1 \end{bmatrix} = \begin{bmatrix} 1 & -0.294 & -0.706 & 0 \\ 0 & 0 & 1 & -1 \end{bmatrix} \tag{46}$$

[C] is the matrix of concentration control coefficients also given in eqn. (20).

Using eqn. 41, we have calculated $[A]^{-1}$ and from our flux ratios we have calculated [D] with eqn. (46). Thus we found [C], with values indistinguishable from those in eqn. (20). We see that, as in the matrix method of Heinrich *et al.* (1977) we here only obtain the concentration control coefficients. Also here the matrix that must be inverted is small. Indeed, this method is virtually identical to the method of Heinrich *et al.* (1977): matrix [A] is identical to the matrix [η] except that its upper row is smaller by a factor v_1, whereas matrix [D] differs from matrix [ϕ] merely in that its first row is a factor v_1 smaller. As disadvantages of these methods, we see (i) the necessity to derive the correspondences between the g and h values and the elasticity coefficients at branch points [eqns. (37-39)], (ii) the possibility that users fail to notice that control coefficients with respect to the α and β values do not directly correspond to the control coefficients with respect to enzyme activities, and (iii) the extra necessity to convert the former to the latter (by deriving matrix [D]). Perhaps it is possible to define a simple algorithm to find matrix [D] and the conversion rules defined by eqns. (37-39). This would certainly improve the user friendliness of the biochemical systems theory method.

Concluding Remarks

In this chapter we have compared four different methods for quantifying control in a metabolic pathway. The methods were applied to the pathway of gluconeogenesis from lactate. All methods require the same type and amount of experimental data. As noted by other authors on the basis of theoretical analyses (Reder in Chapter 7 and Canela, Cascante and Franco in Chapter 11 of this book), we find that the four methods produce different sets of control coefficients. The methods of Savageau (1976) and Heinrich *et al.* (1977) only give

the concentration control coefficients. The matrix method of Fell & Sauro (1987) and Westerhoff & Kell (1987) also generates the control coefficients on the main pathway flux and on a flux ratio. The matrix method of Crabtree & Newsholme produces the control coefficients for all fluxes and metabolite concentrations and may therefore be advantageous. Of course, these differences are minor, because in each case the remaining control coefficients can readily be calculated through additional simple relationships. The numerical values obtained were identical for all four methods.

We encountered considerable difficulties when trying to find out how the methods of Savageau (1976) and Heinrich *et al.* (1977) have to be applied to a real metabolic pathway. This was due to the complexity of the mathematical formulation in which they have been framed. Also, both methods complicate matters somewhat by requiring extra elasticity-like coefficients. In addition, the method of Savageau primarily produces control coefficients with respect to non-molecular properties (complex combinations of enzyme properties rather than single enzyme properties). This problem can be mastered by the introduction of an extra matrix into the equations.

We are satisfied to note that the four calculation methods are simply somewhat different ways of achieving the same end. Suggestions that there are inherent conflicts between the methods appear to be void.

If one is solely interested in obtaining the control coefficients of enzymes on fluxes or concentrations of metabolites we recommend the use of the method of Crabtree & Newsholme (1987) or the method of Fell & Sauro (1985, 1987) and Westerhoff & Kell (1987), as relatively straightforward recipes for filling in the necessary matrices are given. We have a slight preference for the latter method, because it remains closest to the summation and connectivity theorems, which give much insight in the basis for metabolic control distribution. None of the four methods treated here provides a straightforward answer to the fourth control question asked in the introduction, i.e. which regulatory mechanism is most important for pathway control under physiological conditions? We realize that we have not applied biochemical systems theory to its full potential. In contrast to metabolic control theory, biochemical systems theory can be used to obtain information about the dynamic behaviour of a metabolic pathway. In a future study we will evaluate what extra information about the control structure of a metabolic pathway can be obtained by application of biochemical systems theory.

REFERENCES

Burns, J. A., Cornish-Bowden, A., Groen, A. K., Heinrich, R., Kacser, H., Porteous, J. W., Rapoport, S. M., Rapoport, T. A., Stucki, J. W., Wanders, R. J. A. & Westerhoff, H. V. (1985) *Trends Biochem. Sci. 10,* 16

Crabtree, B. & Newsholme, E. A. (1987) *Biochem. J. 247,* 113-120

Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561

Groen, A. K., Van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, T. P. M. & Tager, J. M. (1982) in *Metabolic Compartmentation* (Sies, H., ed.) pp 235-257, Academic Press, New York

Groen, A. K., Vervoorn, R. C., Van der Meer, R. & Tager, J. M. (1983) *J. Biol. Chem. 258,* 14346-14353

Groen, A. K., Roermund, C. W. T., Vervoorn, R. C. & Tager, J. M. (1986) *Biochem. J. 237,* 379-389
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95
Heinrich, R. & Rapoport, T. A. (1975) *BioSystems 7,* 130-136
Heinrich, R., Rapoport, S. M. & Rapoport, T. A. (1977) *Prog. Biophys. Molec. Biol. 32,* 1-82
Hue, L. (1981) *Adv. Enzymol. 52,* 249-331
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Newsholme, E. A. & Crabtree, B. (1985) *Curr. Topics Cell Regul. 25,* 21-76
Rapoport, T. A., Heinrich, R. & Rapoport, S. M. (1976) *Biochem. J. 154,* 449-469
Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221
Savageau M. A. (1969) *J. Theoret. Biol. 25,* 365-369
Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts
Van der Meer, R. & Tager, J. M. (1976) *FEBS Lett. 67,* 36-40
Westerhoff, H. V. & Kell, D. B. (1987) *Biotechnol. Bioeng. 30,* 1101-1107
Westerhoff, H. V., Groen, A. K. & Wanders, R. J. A. (1984) *Biosci. Rep. 4,* 1-22

Part II

MATHEMATICAL ASPECTS OF CONTROL ANALYSIS

Chapter 7

The Structural Approach to Metabolic Control Analysis I: Theoretical Aspects

CHRISTINE REDER

IN THE STUDY of a biochemical system, it is interesting to emphasize its invariants, i.e. the characteristics or properties that depend neither on the state of its environment nor on its internal state, but only on its structure. The models used in metabolic control theory, or more generally in biochemical kinetic theory, can be constructed in two steps: defining first the stoicheiometric reaction scheme, and giving then the expressions of the rate functions of each of the reactions. As the reaction scheme is generally supposed not to depend on the state of the system, it is natural to define the structural properties of a model as those that depend only on this scheme, and not on the rate functions. Then the study of the structural properties of a model can be used into two different ways: either to test directly the reaction network on which a model is based, or to use the network structure of a model, assuming it to be valid, to calculate coefficients that cannot be measured experimentally. This can be done without making any assumptions about the rate functions or about the state of the system. In this paper, we will explain in the context of a simple example how the structural properties of the control coefficients can be constructed, and also how the elasticity coefficients can be calculated from the control coefficients, providing that their structural properties are satisfied. [These results are presented and proved in a general framework in Reder (1988)].

The Model and its First Structural Property: the Conservation Relationships

We suppose that the state of a biochemical system is entirely described by several quantities, classified into internal variables and external parameters. We assume that the internal variables are the concentrations $x_1, x_2, \ldots x_m$ of some metabolites $X_1, X_2, \ldots X_m$ (respectively). We define the concentration vector $\mathbf{x}$ as follows:

Christine Reder ● UER de Mathématique et Informatique, Université de Bordeaux I, 351 cours de la Libération, 33405 Talence Cedex, France

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

$$\mathbf{x} = \begin{bmatrix} x_1 \\ x_2 \\ \vdots \\ x_m \end{bmatrix}$$

To construct the model, we first write the stoicheiometric reaction scheme, ignoring the "external" metabolites whose concentrations are fixed external parameters (shown below as *); we arbitrarily number the r reactions that constitute the scheme and assign them arbitrary directions. For the example we want to study here, m is equal to 3, r to 4 and the reaction scheme is as follows:

$$1.\quad X_3 + * \rightarrow X_1 + X_2$$

$$2.\quad X_1 \rightarrow *$$

$$3.\quad X_1 + X_2 \rightarrow X_3 + *$$

$$4.\quad X_3 \rightarrow X_2$$

To this scheme we associate the scheme matrix **N** of m rows and r columns whose coefficient at column j and row i is the signed stoicheiometric coefficient of the metabolite X_i in the reaction j; hence

$$\mathbf{N} = \begin{bmatrix} 1 & -1 & -1 & 0 \\ 1 & 0 & -1 & 1 \\ -1 & 0 & 1 & -1 \end{bmatrix}$$

The rate of change of the concentration x_i of the metabolite X_i is the sum of the r reaction rates, each weighted by the corresponding stoicheiometric coefficient of X_i. Using v_j to denote the rate of the reaction j and **v** to denote the rate vector, this hypothesis can be expressed as:

$$\frac{d\mathbf{x}}{dt} = \mathbf{N}\mathbf{v}$$

where the rate vector **v** is a function of the concentration vector **x** and of the external parameter vector $\boldsymbol{\mu}$. We get here:

$$\frac{d}{dt}\begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix} = \begin{bmatrix} 1 & -1 & -1 & 0 \\ 1 & 0 & -1 & 1 \\ -1 & 0 & 1 & -1 \end{bmatrix} \begin{bmatrix} v_1(\mathbf{x};\boldsymbol{\mu}) \\ v_2(\mathbf{x};\boldsymbol{\mu}) \\ v_3(\mathbf{x};\boldsymbol{\mu}) \\ v_4(\mathbf{x};\boldsymbol{\mu}) \end{bmatrix}$$

Note that for any choice of the rate function **v**, one has:

$$\frac{d}{dt}(x_2 + x_3) = 0$$

which means that the quantity $(x_2 + x_3)$ remains constant. This linear conservation relationship is structural, as a consequence of a property of the scheme matrix **N**: the sum of its two last rows is indeed zero.

More generally, one can construct all the structural linear conservation relationships as follows: suppose that the first m_0 rows of **N** are independent, and that the $(m - m_0)$ others are linear combinations of these first m_0 rows. The matrix **N** can then be decomposed as

$$\mathbf{N} = \mathbf{L}\,\mathbf{N}_R$$

where all the rows of $\mathbf{N}_R$ are independent and the matrix **L** has the following form:

$$\mathbf{L} = \begin{bmatrix} \mathbf{I}_{m_0} \\ \\ \mathbf{L}_0 \end{bmatrix}$$

where $\mathbf{I}_{m_0}$ denotes the identity matrix of size m_0. The structural conservation relationships are linear combinations of the $(m - m_0)$ independent relationships described by the matrix equality

$$\frac{d}{dt}([-\mathbf{L}_0 \;\; \mathbf{I}_{m-m_0}]\mathbf{x}) = \mathbf{0}$$

For the example, the decomposition of **N** is as follows:

$$\begin{bmatrix} 1 & -1 & -1 & 0 \\ 1 & 0 & -1 & 1 \\ -1 & 0 & 1 & -1 \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ 0 & 1 \\ \text{---} & \text{---} \\ 0 & -1 \end{bmatrix} \begin{bmatrix} 1 & -1 & -1 & 0 \\ 1 & 0 & -1 & 1 \end{bmatrix}$$

$$\mathbf{N} \quad = \quad \mathbf{L} \quad \cdot \quad \mathbf{N}_R$$

The matrix $\mathbf{L}_0$ is as follows:

$$\mathbf{L}_0 = [0 \;\; -1]$$

and the unique conservation relationship is $(x_2 + x_3)$ = constant.

Control Coefficients

Suppose that $\boldsymbol{\sigma}^\circ$ is a steady-state concentration vector of a system for the value $\boldsymbol{\mu}^\circ$ of the external parameter vector. Variations of this steady state can be induced by perturbations both of the external parameters and of the internal state itself. The first type of perturbation acts on the rate function **v**. The second type of perturbation can be easily understood from the following example: adding some quantity of X_2 or X_3 metabolite the "total concentration" $(x_2 + x_3)$ will be modified, and hence the steady state of the system will also vary; on the other hand one can anticipate that by adding any quantity of X_1 metabolite nothing will

change, provided of course that one waits until a steady state is reached again.

We will now explain with the example how one can associate these two types of perturbations with two types of "control coefficients". Suppose for simplicity that there are as many external parameters λ_k as the number r of reactions, and that λ_k acts specifically on the rate function v_k. For the values $\lambda_1^\circ, \lambda_2^\circ, \lambda_3^\circ, \lambda_4^\circ$ of the external parameters, $\boldsymbol{\sigma}^\circ$ is a steady-state concentration vector if

$$\mathbf{Nv} = \begin{bmatrix} 1 & -1 & -1 & 0 \\ 1 & 0 & -1 & 1 \\ -1 & 0 & 1 & -1 \end{bmatrix} \begin{bmatrix} v_1(\boldsymbol{\sigma}^\circ; \lambda_1^\circ) \\ v_2(\boldsymbol{\sigma}^\circ; \lambda_2^\circ) \\ v_3(\boldsymbol{\sigma}^\circ; \lambda_3^\circ) \\ v_4(\boldsymbol{\sigma}^\circ; \lambda_4^\circ) \end{bmatrix}$$

One can suppose that for every parameter vector $\boldsymbol{\lambda}$ sufficiently close to $\boldsymbol{\lambda}^\circ$ and from every initial concentration state $\boldsymbol{\chi}$ sufficiently close to $\boldsymbol{\sigma}^\circ$, the internal state of the system will reach asymptotically a steady state denoted by $\boldsymbol{\sigma}(\boldsymbol{\chi}, \boldsymbol{\lambda})$. The steady-state flux function $\mathbf{F}$ is defined in a natural way as follows:

$$\mathbf{F}(\boldsymbol{\chi}; \boldsymbol{\lambda}) = \mathbf{v}(\boldsymbol{\sigma}(\boldsymbol{\chi}; \boldsymbol{\lambda}); \boldsymbol{\lambda})$$

With perturbations of the external parameters one can associate the classical (but simplified) control coefficient matrices Γ and $\mathbf{C}$:

- Γ is the m-row r-column matrix whose coefficient at row i and column k represents the control of the step k on the steady-state concentration of the metabolite X_i, and is defined as follows:

$$\Gamma_{ik} = \frac{\partial \sigma_i}{\partial \lambda_k} \Big/ \frac{\partial v_k}{\partial \lambda_k}$$

- $\mathbf{C}$ is the r-row r-column matrix whose coefficient at row i and column k represents the control of the step k on the steady-state flux F_i, and is defined as follows:

$$C_{ik} = \frac{\partial F_i}{\partial \lambda_k} \Big/ \frac{\partial v_k}{\partial \lambda_k}$$

all the partial derivatives being calculated at the point $(\boldsymbol{\sigma}^\circ; \boldsymbol{\lambda}^\circ)$.

We can also define matrices $\mathbf{D}_{\boldsymbol{\chi}}\boldsymbol{\sigma}$ and $\mathbf{D}_{\boldsymbol{\chi}}\mathbf{F}$ to represent the variations of the steady state or steady-state fluxes induced by perturbations of the internal state $\boldsymbol{\sigma}^\circ$:

- $\mathbf{D}_{\boldsymbol{\chi}}\boldsymbol{\sigma}$ is an m-row m-column matrix whose coefficient at row i and column k represents the variation of the steady-state concentration of the metabolite X_i induced by a perturbation of the concentration of X_k:

$$(\mathbf{D}_{\chi}\boldsymbol{\sigma})_{ik} = \frac{\partial \sigma_i}{\partial \chi_k}$$

- $\mathbf{D}_{\chi}\mathbf{F}$ is an r-row m-column matrix whose coefficient at row i and column k represents the variation of the steady-state flux F_i induced by a perturbation of the concentration of X_k:

$$(\mathbf{D}_{\chi}\mathbf{F})_{ik} = \frac{\partial F_i}{\partial \chi_k}$$

Structural Properties of the Control Matrices

We have already detailed elsewhere (Reder, 1986, 1988) how these four control matrices can be calculated from $\mathbf{N}_R$, $\mathbf{L}$ and the elasticity matrix $\mathbf{D}_x\mathbf{v}$. We want here to point out the structural properties of the control matrices and how they can be used. Recall that these properties only depend on the scheme matrix $\mathbf{N}$, and not on the rate function $\mathbf{v}$; in particular, they do not depend on the elasticity coefficients.

We need to introduce the matrix $\mathbf{K}$, whose columns constitute a basis of the kernel of $\mathbf{N}_R$; we may choose in our case:

$$\mathbf{K} = \begin{bmatrix} 1 & 1 \\ 0 & 1 \\ 1 & 0 \\ 0 & -1 \end{bmatrix}$$

Suppose now that $\boldsymbol{\sigma}^\circ$ is a non-singular steady state for the external parameter value $\boldsymbol{\lambda}^\circ$ [this mathematical hypothesis is not very strong, as shown in Reder (1986, 1988)]; one can prove that all the structural properties of the matrices Γ, $\mathbf{C}$, $\mathbf{D}_{\chi}\boldsymbol{\sigma}$ and $\mathbf{D}_{\chi}\mathbf{F}$ are consequences of the following:

- (*a*) $[-\mathbf{L}_0 \;\; \mathbf{I}_{m-m_0}]\,\Gamma = 0, \quad \Gamma\,\mathbf{K} = 0 \quad \text{and} \quad \text{rank}\,(\Gamma) = m-m_0$
- (*b*) $\mathbf{N}_R\,\mathbf{C} = 0 \quad \text{and} \quad \mathbf{C}\,\mathbf{K} = \mathbf{K}$
- (*c*) $[-\mathbf{L}_0 \;\; \mathbf{I}_{m-m_0}]\,\mathbf{D}_{\chi}\boldsymbol{\sigma} = [-\mathbf{L}_0 \;\; \mathbf{I}_{m-m_0}] \quad \text{and} \quad \mathbf{D}_{\chi}\boldsymbol{\sigma}\,\mathbf{L} = 0$
- (*d*) $\mathbf{N}_R\,\mathbf{D}_{\chi}\mathbf{F} = 0 \quad \text{and} \quad \mathbf{D}_{\chi}\mathbf{F}\,\mathbf{L} = 0$

Let us translate and comment on the properties (*a*) of the control matrix Γ of our example. In this case, Γ is a 3-row 4-column matrix. The first property of (*a*) becomes:

$$[\,0 \quad 1 \quad 1\,]\,\Gamma = 0$$

and shows that the two last rows of Γ are opposite. This property reflects the fact that the

"total concentration" (x_2+x_3) does not change when one perturbs any external parameter. From the second (a)-property we get the generalized "summation relationships" between the concentration control coefficients, which are discussed in Mazat & Reder (1988) and Reder (1988):

$$\Gamma \begin{bmatrix} 1 & 1 \\ 0 & 1 \\ 1 & 0 \\ 0 & -1 \end{bmatrix} = 0$$

The third column of Γ is then the opposite of the first one, and the last column is the sum of the two first ones. Hence, the matrix has necessarily the following form:

$$\Gamma = \left[\begin{array}{cc:cc} \alpha & \beta & -\alpha & \alpha+\beta \\ \gamma & \delta & -\gamma & \gamma+\delta \\ \hdashline -\gamma & -\delta & \gamma & -\gamma-\delta \end{array}\right]$$

Therefore, only four coefficients of Γ, for example α, β, γ and δ, suffice to calculate all the others. The rank condition of (a) is more technical, and associated with the non-singularity of $\boldsymbol{\sigma}^{\circ}$; it is equivalent to the invertibility of the 2 by 2 matrix composed by the coefficients α, β, γ and δ. We will use this property in the last section of this paper.

Properties (b-d) could be commented on in the same way. They can also be expressed as relationships between the rows or the columns of the matrices $\mathbf{C}$, $\mathbf{D}_{\chi}\boldsymbol{\sigma}$ and $\mathbf{D}_{\chi}\mathbf{F}$. Their use reduces the number of independent coefficients of these matrices. We can summarize these properties as follows:

$$\mathbf{C} = \begin{bmatrix} a & b & 1-a & 1+a+b \\ c & d & -c & 1+c+d \\ a-c & b-d & 1-a+c & a+b-c-d \\ -c & -d & c & -1-c-d \end{bmatrix}$$

$$\mathbf{D}_{\chi}\boldsymbol{\sigma} = \begin{bmatrix} 0 & \tau & \tau \\ 0 & \xi & \xi \\ 0 & 1-\xi & 1-\xi \end{bmatrix}$$

$$\mathbf{D}_{\chi}\mathbf{F} = \begin{bmatrix} 0 & f & f \\ 0 & g & g \\ 0 & f-g & f-g \\ 0 & -g & -g \end{bmatrix}$$

Notice that in these two last matrices the first column is zero: this means that if the internal state of the system is perturbed by adding a quantity of the X_1 metabolite, the system returns to the steady state in which it was before the perturbation. Notice also that the two last columns of $\mathbf{D}_{\chi}\boldsymbol{\sigma}$ or $\mathbf{D}_{\chi}\mathbf{F}$ are equal: this means that adding the X_2 or X_3 metabolite affects the steady state in the same way; we could have expected this as the parameter important for defining the steady state is the "total concentration" ($x_2 + x_3$).

Computation of the Elasticity Matrix from the Control Matrices

It is well known that the four control matrices Γ, **C**, $\mathbf{D}_{\chi}\boldsymbol{\sigma}$ and $\mathbf{D}_{\chi}\mathbf{F}$ can be calculated from the scheme matrix **N** and the elasticity matrix $\mathbf{D}_{\chi}\mathbf{v}$, i.e. the matrix whose coefficient at row *i* and column *j* is:

$$(D_x v)_{ij} = \frac{\partial v_i}{\partial x_j}$$

Conversely, if Γ, **C**, $\mathbf{D}_{\chi}\boldsymbol{\sigma}$ and $\mathbf{D}_{\chi}\mathbf{F}$ are matrices that satisfy the structural properties (*a-d*) of the last section, it can be proved that there exists a rate function **v** such that these matrices are the four control matrices associated to a steady state of the system. Moreover, the associated elasticity matrix $\mathbf{D}_x\mathbf{v}$ is entirely determined from these control matrices; it is actually the unique *r*-row *m*-column matrix solution of:

$$\mathbf{D}_x\mathbf{v}\,\Gamma = \mathbf{C} - \mathbf{I}_r \quad \text{and} \quad \mathbf{D}_x\mathbf{v}\,\mathbf{D}_{\chi}\boldsymbol{\sigma} = \mathbf{D}_{\chi}\mathbf{F}$$

Let us apply this result to our model reaction scheme. For a choice of coefficients (α, β, γ, δ), (*a, b, c, d*), (τ, ξ), (*f, g*), let Γ, **C**, $\mathbf{D}_{\chi}\boldsymbol{\sigma}$ and $\mathbf{D}_{\chi}\mathbf{F}$ be the matrices defined in the previous section. They are the four control matrices associated with the scheme matrix **N** and the elasticity matrix $\mathbf{D}_x\mathbf{v}$, and this elasticity matrix can be calculated solving the system indicated above. This system can be simplified, as the columns of the matrices Γ or $\mathbf{D}_{\chi}\boldsymbol{\sigma}$ are not independent; it can be reduced to the following:

$$\mathbf{D}_x\mathbf{v}\begin{bmatrix} \alpha & \beta & \tau \\ \gamma & \delta & \xi \\ -\gamma & -\delta & 1-\xi \end{bmatrix} = \begin{bmatrix} a\text{-}1 & b & f \\ c & d\text{-}1 & g \\ a\text{-}b & c\text{-}d & f\text{-}g \\ -c & -d & -g \end{bmatrix}$$

This system can be solved for any choice of the coefficients, provided that ($\alpha\delta$ - $\beta\gamma$) is non-zero; but this condition is precisely the rank property imposed on Γ from structural condition (*a*).

This calculation of the elasticity coefficients from the control coefficients is maybe not of great interest from an experimental point of view. Nevertheless, it ensures that all the structural properties of the control matrices can actually be derived from the (*a-d*) properties described in the previous section.

REFERENCES

Mazat, J.-P. & Reder, C. (1988), *Aspects Géométriques de la Théorie du Contrôle du Métabolisme* (Mazat, J.-P. & Reder, C., eds.), Bordeaux

Reder, C. (1986) *Mimodrame mathématique sur les systèmes biochimiques*, Université Bordeaux I. Mathématiques Appliquées. Publication N° 8608

Reder, C. (1988) *J. theor. Biol. 135*, 175-201

Chapter 8

The Structural Approach to Metabolic Control Analysis II: Geometrical Aspects

JEAN-PIERRE MAZAT, CHRISTINE REDER and T. LETELLIER

IN THE GENERAL framework of metabolic control theory (Kacser & Burns, 1973; Heinrich & Rapoport, 1974), Reder (1986, 1988) has described a general method that provides a way of analysing the sensitivity of a metabolic system to perturbation of the environment or of its internal state. This method extends and generalizes the matrix method proposed by Fell & Sauro (1985) and Westerhoff & Chen (1984).

One of the most important ideas of the method is to keep apart what belongs to the structure of the network and what belongs to the velocity functions of the individual steps. We shall describe here some geometrical aspects of the method and to illustrate them with simple examples.

Definitions and Notation

The structure of the metabolic network is expressed as a matrix **N**, the columns of which are the stoicheiometric coefficients of the reactions of the network. We will initially suppose that the rank of the **N** matrix is maximum. In this case, there is no conservation relationships between the concentrations x_i. We shall see in Example 3 (Fig. 1) how to approach the general case where this supposition does not hold.

With this definition, the evolution of the metabolite concentrations x_i ($1 \leq i \leq m$) can be written as follows:

$$\frac{d\mathbf{x}}{dt} = \mathbf{N} \cdot \mathbf{V} \tag{1}$$

where **V** represents the column of the r rates $v_i(x_j, m)$, **x** the column of the m metabolite

Jean-Pierre Mazat and T. Letellier ● Institut de Biochimie Cellulaire et de Neurochimie, Centre National de la Recherche Scientifique et Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France ✦ Christine Reder ● UER de Mathématique et Informatique, Université de Bordeaux I, 351 cours de la Libération, 33405 Talence Cedex, France

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

concentrations x_i, and m the set of external parameters (enzyme concentrations, inhibitors that are not metabolites of the network, K_m, etc.). In Fig. 1 we give some examples of the construction of the matrix **N**. These examples will be studied in the remainder of the chapter.

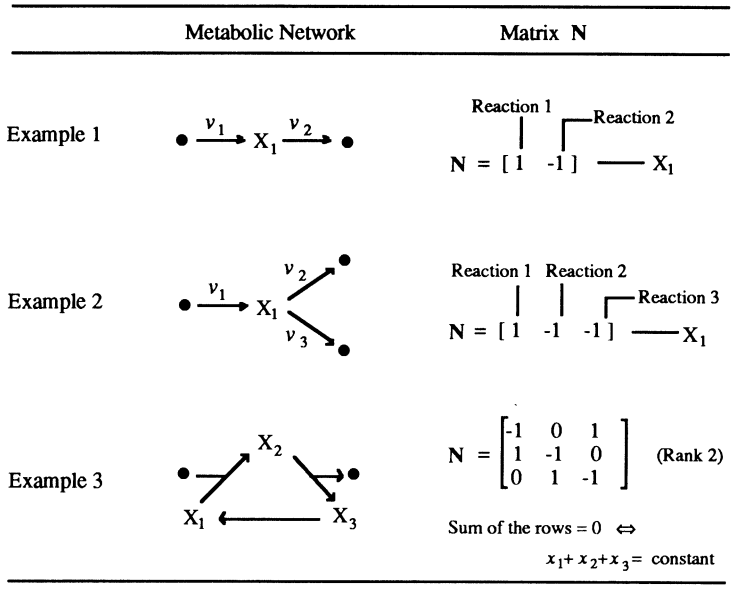


Figure 1. Examples of metabolic networks and their associated **N** matrices.

The Steady State

Let μ_o be a set of external parameters. Let σ_o be a steady state of the system for this value μ_o of the parameters, so that:

$$\mathbf{N} \cdot \mathbf{V}\{[\sigma_0(\mu_0)], \mu_0\} = 0 \tag{2}$$

Let $\mathbf{J}_o$ be the column of steady-state fluxes, i. e.

$$\mathbf{J}_0 = \mathbf{V}\{[\sigma_0(\mu_0)], \mu_0\} \tag{3}$$

Eqn. (2) implies that

$$\mathbf{J}_0 \in \mathrm{Ker}(\mathbf{N}) \tag{4}$$

where Ker(**N**) is the kernel of **N**, i.e. the vector space:

$$\mathrm{Ker}(\mathbf{N}) = \{w \in \mathbb{R}^r / \mathbf{N}w = 0\}$$

On the other hand

$$\mathbf{J}_0 \in \vartheta(\mu_0) \tag{5}$$

where $\vartheta(\mu_o)$ denotes the set of rate vectors associated with the value μ_o of the parameters:

$$\vartheta(\mu_0) = \{\mathbf{V}(x_i, \mu_0)/x_i \in \mathbf{R}^m\}$$

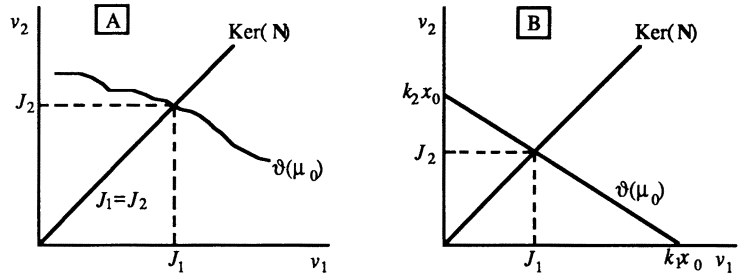


Figure 2. Example 1 from Fig. 1.

In the r-dimensional space of the rates, the steady states (if any) are situated at the intersection of the subset Ker(N) and $\vartheta(\mu_o)$. This point is illustrated in Fig. 2A, where we use Example 1. In this example, the vectors

$$\begin{bmatrix} v_1 \\ v_2 \end{bmatrix} \text{ of } \mathrm{Ker}(\mathbf{N}) \text{ are defined by } \begin{bmatrix} 1 & -1 \end{bmatrix} \begin{bmatrix} v_1 \\ v_2 \end{bmatrix} = 0, \text{ i.e. } v_1 - v_2 = 0$$

In the rate plane, Ker(N) is the first bisectrix. $\vartheta(\mu_0)$ is defined by the fact that in the metabolic pathway, the x_1 value is the same for v_1 and v_2, i.e. by eliminating x_1 between the rate equations of v_1 and v_2. In order to illustrate that point, we have chosen two very simple rate equations:

$$v_1 = k_1(x_0 - x_1) \quad \text{and} \quad v_2 = k_2 x_1$$

Elimination of x_1 gives the equation of $\mathbf{J}(x_0, k_1, k_2)$:

$$v_1 = k_1 x_0 - v_2 k_1/k_2$$

This is represented in Fig. 2B.

Perturbation of the Steady State

Let us explain the determination of the control coefficient matrix for Example 1. A control coefficient correlates a change in a flux with a change in the rates. A perturbation of the rates can be defined in Example 1 by a vector:

$$\delta v = \begin{bmatrix} \delta v_1 \\ \delta v_2 \end{bmatrix}$$

The change in the fluxes are also defined by a vector:

$$\delta J = \begin{bmatrix} \delta J_1 \\ \delta J_2 \end{bmatrix}$$

Let us recall that the fluxes J_1 and J_2 are defined by $J_1 = v_1$ and $J_2 = v_2$ at steady state. The control coefficient matrix **C'** is the matrix that couples the two vectors:

$$\delta \mathbf{J} = \mathbf{C}' \delta \mathbf{v} \tag{6}$$

The change in the rate will shift the space $\vartheta(x_0, k_1, k_2)$ [$\vartheta(\mu_0)$ for the sake of simplicity) to a new space: $\vartheta(x'_0, k'_1, k'_2)$ [$\vartheta(\mu'_0)$ for the sake of simplicity). As the perturbations are small these spaces can be locally identified with their tangents (Fig. 3).

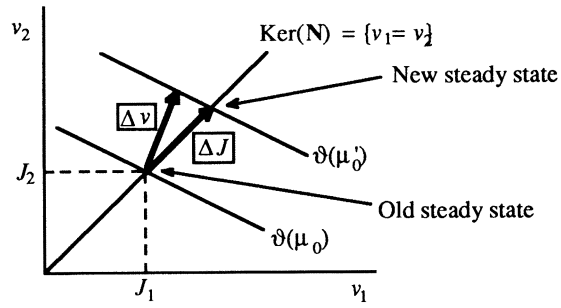


Figure 3. Perturbation of the steady state of Fig. 2B (Example 1 of Fig. 1)

The new steady state will be at the intersection of the new space $\vartheta(\mu'_0)$ (or its tangent) always with Ker(**N**) so that $\delta\mathbf{J}$ appears as the projection of δv on Ker(**N**) in the direction of the tangent to the space $\vartheta(\mu'_0)$. This is a general property and has two important consequences which come from two well-known properties of the projections (Fig. 4). First, if we take a perturbation vector δv in the space on which we project, here Ker(**N**), we obtain the identity; this will give us structural relationships (and in particular the summation relationships). Second, if we take a perturbation vector δv in the direction of the projection we will obtain zero; this will give us the connectivity relationships.

Let us apply these concepts to Example 1. A vector of the space on which we project is, for instance, the vector $\begin{bmatrix} 1 \\ 1 \end{bmatrix}$ and a vector of the tangent space is obviously $\begin{bmatrix} \frac{\partial v_1}{\partial x_1} \\ \frac{\partial v_2}{\partial x_1} \end{bmatrix} = \begin{bmatrix} \varepsilon_1^1 \\ \varepsilon_1^2 \end{bmatrix}$, so that we can write:

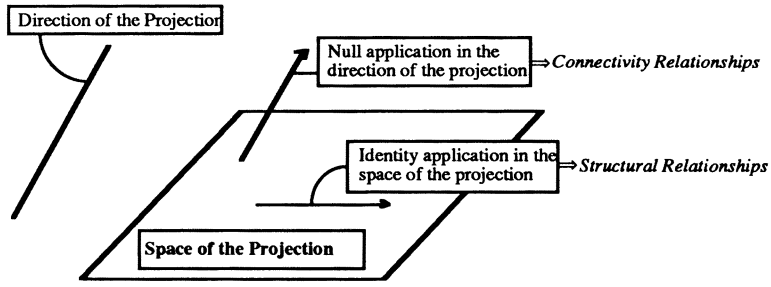


Figure 4. The matrix of the flux control coefficients is the matrix of a *projection.*

$$\begin{bmatrix} C'_{11} & C'_{12} \\ C'_{21} & C'_{22} \end{bmatrix} \begin{bmatrix} 1 & \varepsilon'^{1}_{1} \\ 1 & \varepsilon'^{2}_{1} \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ 1 & 0 \end{bmatrix}$$

$$\mathbf{C'} \quad \times \quad \mathbf{B'} \quad = \quad \mathbf{A'} \tag{7}$$

In this example the two rows of the **C'** matrix are identical because $J_1 = J_2$

We can use now eqn. (7) in two different ways. First it allows us to calculate, numerically or formally, the control coefficients **C'** by inverting the matrix **B'** (when this is possible; we shall treat in Example 3 the case where this is not possible): $\mathbf{C'} = \mathbf{A'}\,\mathbf{B'}^{-1}$. In our case, we obtain the following well-known result:

$$\mathbf{B'}^{-1} = \frac{1}{\varepsilon'^{2}_{1} - \varepsilon'^{1}_{1}} \begin{bmatrix} \varepsilon'^{2}_{1} & -\varepsilon'^{1}_{1} \\ -1 & 1 \end{bmatrix} \tag{8}$$

so that:

$$C'_{11} = C'_{21} = \frac{\varepsilon'^{2}_{1}}{\varepsilon'^{2}_{1} - \varepsilon'^{1}_{1}} \quad \text{and} \quad C'_{12} = C'_{22} = \frac{-\varepsilon'^{1}_{1}}{\varepsilon'^{2}_{1} - \varepsilon'^{1}_{1}} \tag{9}$$

Eqn. (7) also allows automatic derivation of the structural (summation) and connectivity relationships. By multiplying the first row of **C'** by the first column of **B'** we obtain the classical summation relationship:

$$C'_{11} \cdot 1 + C'_{21} \cdot 1 = 0 \tag{10}$$

and by multiplying the first row of **C'** by the second column of **B'** we obtain the classical connectivity relationship:

$$C'_{11} \cdot \varepsilon'^{1}_{1} + C'_{21} \cdot \varepsilon'^{2}_{1} = 0 \tag{11}$$

Other Examples

Example 2 and its **N** matrix are described in Fig. 1. The control coefficient matrix is as follows:

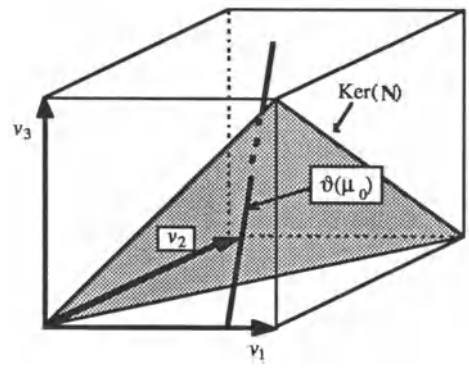


Figure 5. Representation of Ker(**N**) and $\vartheta(\mu_o)$ for Example 2

$$\mathbf{C'} = \begin{bmatrix} C'_{11} & C'_{12} & C'_{13} \\ C'_{21} & C'_{22} & C'_{23} \\ C'_{31} & C'_{32} & C'_{33} \end{bmatrix}$$

Contrary to the previous case the rows are not identical. We shall first determine the kernel of **N**, Ker(**N**). A vector in Ker(**N**) is such that:

$$\mathbf{N}\begin{bmatrix} v_1 \\ v_2 \\ v_3 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix}, \text{ i.e. } v_1 - v_2 - v_3 = 0 \tag{12}$$

Eqn. (12) is the equation of a plane (dimension = 2). A parametric representation of this plane is:

$$v_2 = av_1 \quad \text{and} \quad v_3 = (1 - a)v_1$$

It appears in grey in Fig. 5. Two independent vectors of the plane are $\begin{bmatrix} 1 \\ 1 \\ 0 \end{bmatrix}$ and $\begin{bmatrix} 1 \\ 0 \\ 1 \end{bmatrix}$. On the other hand the space of the allowable rate (by elimination of x_1) has a dimension equal to 1. To see this we have chosen the following very simple rate equations:

$$v_1 = k_1 (x_0 - x_1)$$

$$v_2 = k_2 x_1$$

$$v_3 = k_3 x_1$$

$\vartheta(\mu_o)$ is then a straight line intersecting Ker(**N**) at the steady state. The tangent plane to $\vartheta(\mu_o)$ is given by the vector: $\begin{bmatrix} \varepsilon'^1_1 \\ \varepsilon'^2_1 \\ \varepsilon'^3_1 \end{bmatrix}$. In that case eqn. (7) becomes:

$$\begin{bmatrix} C'_{11} & C'_{12} & C'_{13} \\ C'_{21} & C'_{22} & C'_{23} \\ C'_{31} & C'_{32} & C'_{33} \end{bmatrix} \begin{bmatrix} 1 & 1 & \varepsilon'^{1}_{1} \\ 1 & 0 & \varepsilon'^{2}_{1} \\ 0 & 1 & \varepsilon'^{3}_{1} \end{bmatrix} = \begin{bmatrix} 1 & 1 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \end{bmatrix}$$

$$\mathbf{C'} \quad \times \quad \mathbf{B'} \quad = \quad \mathbf{A'}$$

As previously, one can now (i) invert the **B'** matrix in order to obtain **C'**, and (ii) make the formal products to obtain the structural and the connectivity relationships. For instance:

Row 1 x Column 1:	$C'_{11} + C'_{12} = 1$	which leads to	$aC_{11} + C_{12} = a$
Row 1 x Column 2:	$C'_{11} + C'_{13} = 1$	which leads to	$(1-a)C_{11} + C_{13} = (1-a)$

where $C_{ij} = C'_{ij} \cdot v_j/F_i$ corresponds to the logarithmic derivatives. The sum of these two relations gives the well known summation relationship:

$$C_{11} + C_{12} + C_{13} = 1$$

The other structural relationships can be deduced in the same way.

We can now write the connectivity relationships as follows:

Row 1 x Column 3: $C'_{11}\varepsilon'^{1}_{1} + C'_{12}\varepsilon'^{2}_{1} + C'_{13}\varepsilon'^{3}_{1} = 0$

which is equivalent to $C_{11}\varepsilon^{1}_{1} + C_{12}\varepsilon^{2}_{1} + C_{13}\varepsilon^{3}_{1} = 0$

with $\varepsilon''^{i}_{j} = \varepsilon^{i}_{j} \cdot v_i/x_j$(logarithmic derivatives). Two other relationships can be deduced in the same way.

Example 3

The metabolic network of Example 3 and the associated matrix **N** are described in Fig.1. As indicated in this figure, the sum of the rows of the matrix **N** is equal to zero; this indicates a conservation relationship between concentrations of the metabolites:

$$x_1 + x_2 + x_3 = \text{constant}$$

Eqn. (7) does not allow calculation of the control coefficients because the matrix **B'** is no longer invertible. We are led (see Reder, 1986, 1988) to extract from **N** a matrix $\mathbf{N_R}$ of maximum rank, here 2 (number of rows minus number of relationships between the rows). For example, we can take the two first rows of the matrix **N** as $\mathbf{N_R}$:

$$\mathbf{N_R} = \begin{bmatrix} -1 & 0 & 1 \\ 1 & -1 & 0 \end{bmatrix}$$

We then define the matrix $\mathbf{L}_0$ such that the last row of $\mathbf{N}$ is equal to $\mathbf{L}_0\mathbf{N}_R$:

$$[0 \quad 1 \quad -1] = \mathbf{L}_0\mathbf{N}_R$$

This is always possible as the last row is a linear combination of the previous ones. We obtain:

$$\mathbf{L}_0 = [-1 \quad -1]$$

Thus, we can write $\mathbf{N} = \mathbf{L}\mathbf{N}_R$ with $\mathbf{L} = \begin{bmatrix} 1 & 0 \\ 0 & 1 \\ -1 & -1 \end{bmatrix}$. In our example, the kernel of $\mathbf{N}$ is defined by:

$$\begin{bmatrix} -1 & 0 & 1 \\ 1 & -1 & 0 \\ 0 & 1 & -1 \end{bmatrix} \begin{bmatrix} v_1 \\ v_2 \\ v_3 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix}, \quad \text{i.e.} \quad v_1 = v_2 = v_3$$

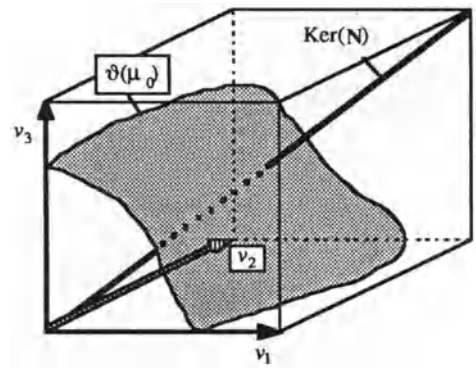


Figure 6. Representation of Ker(**N**) and $\vartheta(\mu_o)$ for Example 3

The dimension of Ker(N) is 1 (see Fig. 6) and a vector in this vector space is $\begin{bmatrix} 1 \\ 1 \\ 1 \end{bmatrix}$. We define the matrix $\mathbf{E}'$ of the elasticities:

$$\mathbf{E}' = \begin{bmatrix} \varepsilon'^1_1 \varepsilon'^1_2 \varepsilon'^1_3 \\ \varepsilon'^2_1 \varepsilon'^2_2 \varepsilon'^2_3 \\ \varepsilon'^3_1 \varepsilon'^3_2 \varepsilon'^3_3 \end{bmatrix}$$

One can define now the matrix $\mathbf{C}'$ as the matrix of a projection on $\mathrm{Ker}(\mathbf{N}_R) = \mathrm{Ker}(\mathbf{N})$ in the direction of the tangent space of $\vartheta(\mu_o)$.

The manifold $\vartheta(\mu_o)$ depends on the three variables x_1, x_2 and x_3 bound by the relation-

ship $x_1 + x_2 + x_3 =$ constant. $\vartheta(\mu_o)$ is thus a surface of dimension 2. Two independent vectors of its tangent plane are obtained by the product:

$$\mathbf{E'L} = \begin{bmatrix} \varepsilon'^1_1 - \varepsilon'^1_3 & \varepsilon'^1_2 - \varepsilon'^1_3 \\ \varepsilon'^2_1 - \varepsilon'^2_3 & \varepsilon'^2_2 - \varepsilon'^2_3 \\ \varepsilon'^3_1 - \varepsilon'^3_3 & \varepsilon'^3_2 - \varepsilon'^3_3 \end{bmatrix}$$

Thus we can now write:

$$\begin{bmatrix} C'_{11} & C'_{12} & C'_{13} \\ C'_{21} & C'_{22} & C'_{23} \\ C'_{31} & C'_{32} & C'_{33} \end{bmatrix} \begin{bmatrix} 1 & \varepsilon'^1_1 - \varepsilon'^1_3 & \varepsilon'^1_2 - \varepsilon'^1_3 \\ 1 & \varepsilon'^2_1 - \varepsilon'^2_3 & \varepsilon'^2_2 - \varepsilon'^2_3 \\ 1 & \varepsilon'^3_1 - \varepsilon'^3_3 & \varepsilon'^3_2 - \varepsilon'^3_3 \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 1 & 0 & 0 \\ 1 & 0 & 0 \end{bmatrix}$$

$$\mathbf{C'} \quad \times \quad [\,\mathbf{K} \qquad \mathbf{E'\cdot L} \qquad] = [\,\mathbf{K} \;\; \mathbf{0} \;\; \mathbf{0}\,] \tag{13}$$

where **K** represents a basis of Ker(**N**). As we did previously, we can use this equality either to calculate the matrix **C'**, i.e. the flux control coefficients or to derive the formal structural and connectivity relationships. For instance:

Row 1 x Column 1 (*Summation relationship*):

$$C'_{11} + C'_{12} + C'_{13} = 1, \text{ which leads to } C_{11} + C_{12} + C_{13} = 1$$

Row 1 x Column 2 (*Connectivity relationship*):

$$C'_{11}(\varepsilon'^1_1 - \varepsilon'^1_3) + C'_{12}(\varepsilon'^2_1 - \varepsilon'^2_3) + C'_{13}\,(\varepsilon'^3_1 - \varepsilon'^3_3) = 0, \text{ which leads to}$$

$$C_{11}\left(\frac{S_1}{v_1}\varepsilon^1_1 - \frac{S_3}{v_1}\varepsilon^1_3\right) + \cdots = 0 \tag{14}$$

where the definitions of the C_{ij} and of the ε^j_i are as above (logarithmic derivatives)

Conclusions

The method for which we have described some geometrical aspects by means of some examples provides a way to determine the control coefficients from the elasticity coefficients, and to derive the structural (summation and others) and connectivity relationships. This method generalizes the previous known methods. It can be applied to any metabolic system: for instance, there can be conservation relationships, the external parameters can act in any manner on the reaction rates (in particular, the parameters do not have to act specifically on one reaction rate). It requires knowledge of the rates and metabolite con-

centrations at the steady state and of the partial derivatives of the rates with respect to the metabolite concentrations:

$$\varepsilon_i^j = \frac{\partial v_j}{\partial x_i}$$

The general equation allowing the determination of the control coefficients or of the various relationships is the eqn. (13), which is derived from the fact that the control coefficient matrix C' is a projection matrix on the kernel of **N** in the direction of the tangent space to $\vartheta(\mu_o)$, a basis of which is given by the columns of the matrix **E'L**. There is an equivalent equation for the control coefficient matrix of the substrate Σ' (see Reder, 1986, 1988).

This method is of course equivalent to the method given by Fell & Sauro (1985) and of Sauro *et al.* (1987) in some particular cases. It generalizes this method to all cases and gives its mathematical basis.

It should be pointed out, in relation to Example 3, that the usual definitions of the control coefficients and of the elasticities with logarithmic derivatives lead to complex relationships involving the rate and the metabolite concentrations values at the steady state [eqn. (14)]. This is not the case when the direct derivatives are used (C' and ε'). Concerning Example 2 it should also pointed out that with this definition there twice as many structural relationships between the C' as there are summation relationships with the C. In simple cases (Example 1) both definitions give the same relationships (and have the same values) but this is not a general relationship and we pose the question of whether the traditional definitions should be maintained.

Note: A computer program allowing automatic derivation of the flux and metabolite control coefficients and the literal structural and connectivity relationships has been developed in our laboratory. Further information may be found in Appendix B of this book.

Acknowledgements: This work was supported by the CNRS (ATP Biologie-Mathématiques) and by the Universités Bordeaux I and Bordeaux II.

REFERENCES

Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Reder, C. (1986) *Mimodrame mathématique sur les systèmes biochimiques.* Université Bordeaux I, Mathématiques Appliquées. Publication N° 8608
Reder, C. (1988) *J. Theor. Biol. 135,* 175-201
Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221
Westerhoff, H. V. & Chen, Y. D. (1984) *Eur. J. Biochem. 142,* 425-430

Chapter 9

Control Coefficients and the Matrix Method

DAVID A. FELL, HERBERT M. SAURO and J. RANKIN SMALL

WHEN WE DEVELOPED the matrix method (Fell & Sauro, 1985), we were aiming to find a quick route for the evaluation of flux control coefficients in terms of elasticities, either algebraically for the general case, or numerically in specific instances where the values of the elasticities were known. Our starting points were the summation and connectivity theorems for flux control coefficients of Kacser & Burns (1973), as these seemed to offer a route to the answer using less information about the pathway than the method of Heinrich & Rapoport (1974). Kacser & Burns (1973) had derived these relationships for a general metabolic system, though the specific example treated in their paper was a linear pathway. Their methods of deriving expressions for flux control coefficients could be applied to other pathways [for example, a branched pathway: Kacser (1983)], but on a case by case argument from first principles.

The basis of our method was a set of simple rules for writing a matrix equation that could be solved for the flux control coefficients by standard algebraic or computational procedures. Although the linear metabolic pathway is simple to treat, it can serve as an introduction to the method. To be more specific, consider the analysis of the pathway of serine biosynthesis in rabbit liver (Fell & Snell, 1988):

$$\text{3-phosphoglycerate} \overset{1}{\rightleftharpoons} \text{phosphoserine} \overset{2}{\longrightarrow} \text{serine} + \text{inorganic phosphate}$$

Here 3-phosphoglycerate can be considered as a source, as it is produced and degraded in carbohydrate metabolism, which is many times more active than this anabolic pathway. Serine can be considered a sink in this instance, as its concentration in liver varies relatively little (LaBaume *et al.*, 1987). The first step shown is actually two separate enzymic steps that cannot be resolved in our analysis because the concentration of the intermediate (phosphohydroxypyruvate) is immeasurably low, but this does not matter greatly as the overall step shown is close to equilibrium (LaBaume *et al.*, 1987). We have, with the flux

David A. Fell ● School of Biological and Molecular Sciences, Oxford Polytechnic, Oxford OX3 0BP, UK ❖ Herbert M. Sauro ● Department of Genetics, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK ❖ J. Rankin Small ● Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

through the pathway designated J (where $J = J_1 = J_2$, since the flux is the same through both steps), and phosphoserine abbreviated Ser*P*:

$$C_1^J + C_2^J = 1 \qquad \text{(Summation theorem)}$$

$$C_1^J \varepsilon_{SerP}^1 + C_2^J \varepsilon_{SerP}^2 = 0 \qquad \text{(Connectivity theorem)}$$

The matrix method involves writing this as follows:

$$\begin{bmatrix} 1 & 1 \\ \varepsilon_{SerP}^1 & \varepsilon_{SerP}^2 \end{bmatrix} \begin{bmatrix} C_1^J \\ C_2^J \end{bmatrix} = \begin{bmatrix} 1 \\ 0 \end{bmatrix}$$

or

$$\mathbf{E} \bullet \mathbf{C} = \mathbf{M}$$

which has the solution:

$$\begin{bmatrix} C_1^J \\ C_2^J \end{bmatrix} = \begin{bmatrix} 1 & 1 \\ \varepsilon_{SerP}^1 & \varepsilon_{SerP}^2 \end{bmatrix}^{-1} \begin{bmatrix} 1 \\ 0 \end{bmatrix} = \frac{1}{\varepsilon_{SerP}^2 - \varepsilon_{SerP}^1} \begin{bmatrix} \varepsilon_{SerP}^2 \\ -\varepsilon_{SerP}^1 \end{bmatrix}$$

or

$$\mathbf{C} = \mathbf{E}^{-1} \bullet \mathbf{M}$$

For rabbit liver under normal conditions, the measured mass-action ratio of the first step (LaBaume *et al.*, 1987) can be used to calculate ε_{SerP}^1 to be -1.43 (Fell & Snell, 1988). The second elasticity, ε_{SerP}^2, can be calculated, from the kinetic rate law describing rat liver phosphoserine phosphatase at the measured intracellular levels of phosphoserine and serine, to be 0.041 (Fell & Snell, 1988). Substituting these values in the equations above gives $C_1^J = 0.03$ and $C_2^J = 0.97$. This simple example is of interest because it shows a pathway where the first two enzymes (combined here as step 1) are close to equilibrium, and the final step is subject to feedback inhibition by the end-product of the pathway, serine. The uncompetitive inhibition of the phosphatase by serine is strong, with $\varepsilon_{Ser}^2 = -0.65$. The effect of serine on the pathway flux can therefore be calculated as the response coefficient (Kacser & Burns, 1973):

$$R_{Ser}^J = C_2^J \varepsilon_{Ser}^2 = -0.63$$

There is no difficulty in generalizing this procedure to any length of linear pathway, in the following manner:

1. Create a matrix with as many columns as reactions, n. Each reaction is associated with a particular column.

2. In each position of the first row enter a 1 to represent the summation theorem.

3. For each of the n - 1 variable metabolites, write a row of the matrix to represent each of the connectivity relationships. Each position on the row represents the effect of the metabolite associated with the row on the reaction associated with the column. If the metabolite has an effect, whether as substrate, product, or effector, an elasticity term is entered; otherwise (as is most often the case) a zero is entered.

This generates the matrix **E**, which is inverted to obtain the flux control coefficients in the first column of the inverse. [The other columns of the inverse contain the concentration control coefficients, as described for example in Sauro *et al.* (1987). However we shall not deal with these here.]

The process of matrix inversion can be carried out according to a set of simple rules to be found in any linear algebra text book. Computer programs exist to perform the inversion symbolically (e.g. general symbolic algebra packages such as REDUCE (Hearn, 1985), or a simple Pascal program by Fell and Sauro), and for numerical solutions, functions and subroutines are widely available for most computer systems in most languages. However, there are other "recipes" for obtaining these solutions that are essentially related to the inversion of the matrix **E** (e.g Hofmeyr, 1986*a*, Giersch, 1988*c*).

The problem of extending our method to a branched pathway was that each branch point adds a reaction without adding a variable metabolite, so the simple procedure above does not generate enough rows in the **E** matrix (which must have as many rows as columns for the equations to be soluble). The case of three enzymes forming a single branch point about one metabolite had been solved by Kacser (1983). We generalized Kacser's approach to show that there was a general constraint on the flux control coefficients in branched pathways: the branch point theorem (Fell & Sauro, 1985). For a system of n reactions and m metabolites, we proposed that the fourth step in writing the **E** matrix would be to write a row containing the coefficients of each of the (n - m -1) branch point equations, which with the summation theorem row and the m connectivity theorem rows gives a square matrix. The terms that appear in the branch point rows correspond to fractional fluxes in the branches, and this is the only stage at which fluxes appear in the matrix method. Our solution to the branch point problem was derived for a specific pathway geometry, and is therefore open to the criticism that it does not guarantee the existence of an appropriate set of branch point relationships for every possible pathway configuration. The rigorous justification of our solution is provided by Reder's (1986, 1988) demonstration that the branch-point relationships are, like the summation theorem, derived from the structural properties of the pathway, in that together they form a basis (multiplied by a scaling matrix) of the null space of the stoicheiometry matrix of any metabolic system that has a steady state. As the number of independent vectors required to form a basis for this null space is of necessity the number of reactions (columns) minus the number of independent metabolites (rows), we can be sure that, whatever the structure, there do exist sufficient branch point equations to form a square **E** matrix. In a pathway with multiple branches, a set of independent branch point equations must be selected, and (for the purposes of our method) none of these must have a non-zero coefficient for the flux control coefficients of enzymes carrying the selected reference flux. Fortunately this abstract requirement can be visualized on the map of the reaction pathway:

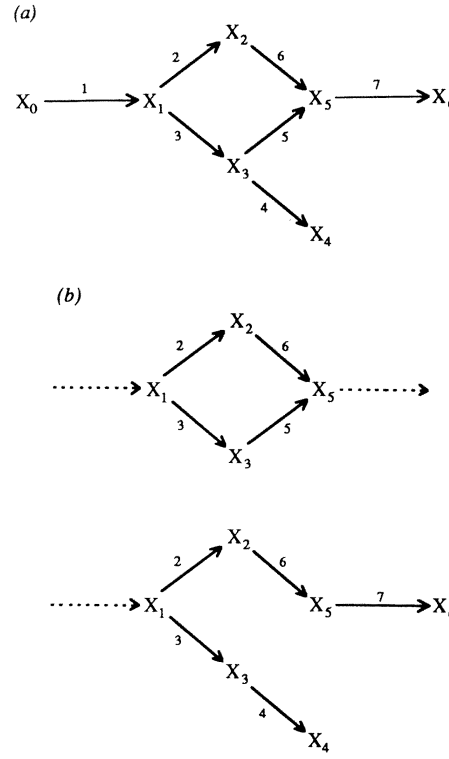


Figure 1. A hypothetical pathway with multiple branches. (*a*) The complete pathway, in which X_0, X_4 and X_6 are external pools. (*b*) The two independent routes traced by the two branch-point equations given in the text.

the set of independent relationships must trace routes between external pools (regardless of the actual reaction direction) such that every reaction, except those in the limb containing the reference flux, appears in at least one route, and no route is duplicated. Thus the pathway shown as Fig. 1*a*, with 7 reactions and 4 independent metabolites, needs two branch-point equations, and a possible choice for these corresponds to the two routes shown as Fig. 1*b*. The form of the equations, and their relationship to choice of reference flux and branch distribution coefficients (Sauro *et al.*, 1987; Westerhoff & Kell, 1987) is described in Small & Fell (1989). The result is as follows:

$$-\frac{J_2}{J_1}\left(C_3^i + C_3^i\frac{J_3}{J_5}\right) + \frac{J_3}{J_1}\left(C_2^i + C_6^i\right) = 0$$

$$-\frac{J_2}{J_1}\left(C_3^i + C_4^i\frac{J_3}{J_4}\right) + \frac{J_3}{J_1}\left(C_2^i + C_6^i\frac{J_2}{J_6} + C_7^i\frac{J_2}{J_7}\right) = 0$$

Note that the pathway in Fig. 1 also illustrates the fact that the endpoint of a route for a branch point equation can be a reconvergence of two arms of a branch to reconstitute the reference flux, instead of an external pool. This is equivalent to a substrate cycle structure, which emphasizes that there is no significant difference between such cycles and branched structures as far as their treatment in the matrix method is concerned. However, we did

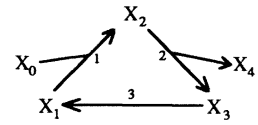


Figure 2. A hypothetical conserved cycle structure. X_0 and X_4 are external pools. The total concentrations of X_1, X_2 and X_3 must remain constant.

distinguish the two cases in our original paper (Fell & Sauro, 1985), since the biochemical significance of the two can be different.

The other major problem in implementing the matrix method arises from the existence of conservation relationships, that is, constraints on the total amount of a group of metabolites that contain a moiety which is not connected to an external pool. Thus, although the system as a whole is open, the total amount of the conserved moiety remains constant on the time scale of the experiment. In these cases, there appear to be more metabolites than required to provide the rows of the **E** matrix. We pointed out (Fell & Sauro, 1985) that the usual form of the connectivity theorem cannot be applied to metabolites belonging to a conserved group, but a modified form could be derived. For each conservation relationship, the number of metabolites for which a row is entered in the **E** matrix is one less than the number of metabolites in the group, and the connectivity relationships that are used each involve the elasticities of one of these metabolites and scaled elasticities of the metabolite that does not contribute a row. Again, it could be argued that our derivation of the modified connectivity relationship was specific to particular configurations of metabolic pathway However, the same modified connectivity relationships arise from the general analysis of Reder (1988; also Reder & Mazat, 1988). Here they are linked to analysis of the stoicheiometry matrix of the system, since the existence of conservation relationships corresponds to lack of independence between the rows of the stoicheiometry matrix. In difficult cases, unambiguous diagnosis of the conservation relationships can be achieved by reduction, or rank analysis, of the stoicheiometry matrix (Hofmeyr, 1986*ab;* Reder, 1988). Details of the writing of the modified connectivity relationships for conserved metabolites, and the best form of the relationship to use when concentration control coefficients are being determined as well, are given in our papers (Fell & Sauro, 1985; Sauro *et al.*, 1987). As a brief example, consider the hypothetical system shown in Fig. 2. The **E** matrix for this pathway is written, according to our rules, as:

$$\begin{bmatrix} 1 & 1 & 1 \\ \varepsilon_1^1 & -\varepsilon_3^2\frac{X_1}{X_3} & \varepsilon_1^3 - \varepsilon_3^3\frac{X_1}{X_3} \\ \varepsilon_2^1 & \varepsilon_2^2 - \varepsilon_3^2\frac{X_2}{X_3} & -\varepsilon_3^3\frac{X_2}{X_3} \end{bmatrix}$$

This leads to the same expressions for the control coefficients as obtained for analysis of this system by Reder & Mazat (1988). The point we wish to emphasize here is that it is only in the scaling factors for elasticities associated with these conserved metabolites that metabol-

ite concentrations explicitly appear in the matrix method, and then only the concentrations of these conserved metabolites.

Apart from our matrix method (Fell & Sauro, 1985), other derivations of matrix expressions for control coefficients have been developed within the sphere of metabolic control analysis (e.g. Reder, 1988; Giersch, 1988*abc;* Westerhoff & Chen, 1984; Cascante *et al.*, 1989; Crabtree & Newsholme, 1987), and there has been continued exposition of the corresponding methodology in biochemical systems theory (Savageau *et al.*, 1987). What can we now perceive as the limitations of our matrix method, and does it have any continuing role? Firstly, the limitations:

1. The proof of the matrix method may not be considered completely rigorous as it is based on arguments derived from consideration of specimen pathway structures. Here the works of Reder (1988) and Cascante *et al.* (1989) are relevant as they show that equations of the form generated by the matrix method can be derived without consideration of the specific structure of the pathway.

2. As more complex pathways are considered, the treatment of conservation relationships and branch points by the rules of the matrix method becomes more difficult (e.g. Small & Fell, 1989). Furthermore, the identification of the conservation relationships is not integrated with the matrix method, but is a separate stage (Hofmeyr, 1986*ab*).

3. The process still involves matrix inversion and multiplication, and this is a barrier for many life scientists, even though computer programs are readily available to carry out these procedures. Furthermore, this algebra can be performed by the same simple "recipes" in every case.

Some of the continuing advantages of the matrix method are as follows:

1. It is a "minimalist" approach, in the sense that it requires only a limited set of metabolite concentrations, some stoicheiometric coefficients, and relative fluxes in branched pathways. Other more rigorous approaches develop equations that include all stoicheiometric coefficients, concentrations and reaction velocities, even though the majority of these will cancel out in the final expressions. Setting up an analysis by the matrix method therefore identifies the quantities that will have to be known or measured experimentally.

2. Most experimental applications of control analysis to date have been on small systems of simple structure, for which the matrix method is fast and easy to use.

3. Even if expressions for the control coefficients are derived by the more rigorous approaches cited previously, the rules of the matrix method, coupled with the theorems of Kacser & Burns (1973), offer a physical interpretation of the terms in the equations.

4. In those cases where it may prove to be necessary, it is relatively simple to modify the matrix method to take account of enzyme-enzyme interaction and other sources of non-

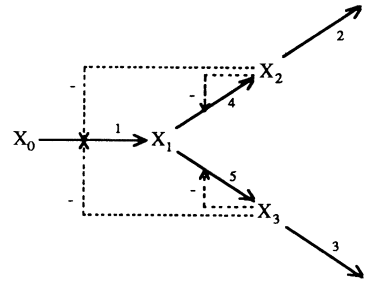


Figure 3. The branched pathway analysed by Savageau *et al.* (1987). The numbers on the arrows correspond to the numbering of the enzymes used by these authors in their metabolic control analysis.

linearity between the rate of a reaction and the amount of an enzyme, as discussed in Chapter 20 of this book by Kacser, Sauro and Acerenza.

For the future, we consider that one way to minimize the disadvantages of the matrix method would be to develop computer programs that will generate and evaluate the expressions for the control coefficients from a simple description of the pathway. Thus the details of the mathematics would be hidden from the user. Such programs are being developed by David Fell in Oxford and Jean-Pierre Mazat in Bordeaux. (See also Appendix B in this book).

The Matrix Method and Biochemical Systems Theory

Chapters 4 and 5 of this book, by Savageau and Voit respectively, are concerned with *biochemical systems theory* and with metabolic control analysis, which they see as a special case of biochemical systems theory. Earlier, in the course of a comparison between biochemical systems theory and metabolic control analysis, they undertook the analysis of a branched pathway (Fig. 3) by both techniques (Savageau *et al.*, 1987). We wish to draw attention to some other aspects of that comparison. The biochemical systems theory approach to this analysis involves writing the matrix **A** of power law coefficients thus:

$$\mathbf{A} = (\mathbf{A}_1 \mid \mathbf{A}_2) = \left[\begin{array}{c|ccc} g_{10} & -h_{11} & (g_{12} - h_{12}) & (g_{13} - h_{13}) \\ 0 & g_{21} & (g_{22} - h_{22}) & 0 \\ 0 & g_{31} & 0 & (g_{33} - h_{33}) \end{array}\right]$$

There are three dependent concentration variables and five enzymic reactions, but in the biochemical systems theory representation, the three metabolites imply six rate parameters (three α and three β parameters). Therefore one of these is redundant (β_1). (There are also grounds for objecting to the definition of a "parameter" of undefinable and inconstant dimensions; see Cornish-Bowden, 1989). In addition, there are eight interactions of the metabolites with the enzymes (as substrates or effectors), but 11 power-law indices in $\mathbf{A}_2$, so

three of these are redundant (h_{11}, h_{12} and h_{13}). The matrix of sensitivities of concentrations to the α rate parameters, $\mathbf{S}_{\alpha}^{X}$ is (Savageau *et al.*, 1987):

$$\mathbf{S}_{\alpha}^{X} = -\begin{bmatrix} -h_{11} & (g_{12} - h_{12}) & (g_{13} - h_{13}) \\ g_{21} & (g_{22} - h_{22}) & 0 \\ g_{31} & 0 & (g_{33} - h_{33}) \end{bmatrix}^{-1}$$

From this, we can derive the matrix expression for the sensitivity of the three fluxes in the system to the three α rate parameters as:

$$\mathbf{S}_{\alpha}^{v} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix} - \begin{bmatrix} 0 & g_{12} & g_{13} \\ g_{21} & g_{22} & 0 \\ g_{31} & 0 & g_{33} \end{bmatrix} \begin{bmatrix} -h_{11} & (g_{12} - h_{12}) & (g_{13} - h_{13}) \\ g_{21} & (g_{22} - h_{22}) & 0 \\ g_{31} & 0 & (g_{33} - h_{33}) \end{bmatrix}^{-1}$$

The corresponding analysis in metabolic control analysis can be carried out extremely simply using the matrix method by forming the matrices **C**, **E** and **M** as before, where:

$$\mathbf{C} = \begin{bmatrix} C_1^1 & C_1^{X_1} & C_1^{X_2} & C_1^{X_3} & C_1^{J_2/J_1} \\ C_2^1 & C_2^{X_1} & C_2^{X_2} & C_2^{X_3} & C_2^{J_2/J_1} \\ C_3^1 & C_3^{X_1} & C_3^{X_2} & C_3^{X_3} & C_3^{J_2/J_1} \\ C_4^1 & C_4^{X_1} & C_4^{X_2} & C_4^{X_3} & C_4^{J_2/J_1} \\ C_5^1 & C_5^{X_1} & C_5^{X_2} & C_5^{X_3} & C_5^{J_2/J_1} \end{bmatrix}$$

$$\mathbf{E} = \begin{bmatrix} 1 & 1 & 1 & 1 & 1 \\ 0 & 0 & 0 & \varepsilon_1^4 & \varepsilon_1^5 \\ \varepsilon_2^1 & \varepsilon_2^2 & 0 & \varepsilon_2^4 & 0 \\ \varepsilon_3^1 & 0 & \varepsilon_3^3 & 0 & \varepsilon_3^5 \\ 0 & \frac{J_2}{J_1} - 1 & \frac{J_2}{J_1} & \frac{J_2}{J_1} - 1 & \frac{J_2}{J_1} \end{bmatrix}$$

$$\mathbf{M} = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 \\ 0 & -1 & 0 & 0 & 0 \\ 0 & 0 & -1 & 0 & 0 \\ 0 & 0 & 0 & -1 & 0 \\ 0 & 0 & 0 & 0 & (J_2/J_1) - 1 \end{bmatrix}$$

Now it is clear that the effect of a change in the activity of enzyme 1, 2 or 3 can be expressed in the biochemical systems theory version as a change in the rate parameters α_1, β_2 and β_3, so that for the variable Y (either a flux or a concentration), we would expect:

$$S(Y, \alpha_1) \equiv C_1^Y; \quad S(Y, \beta_2) \equiv C_2^Y, \quad \text{and} \quad S(Y, \beta_3) \equiv C_3^Y$$

At the operating point of the power-law approximation of biochemical systems theory, where the power-law exponents are instantaneously equivalent to the elasticities as described by Savageau *et al.* (1987), we find that the identical expressions are obtained as expected from the solutions to the matrix equations given above. This was noted by Savageau *et al.*, but they then claimed that the biochemical systems theory analysis was superior because it had the supposed benefits (Voit & Savageau, 1987) of being based on an aggregated power-law representation, whereas the metabolic control analysis was equivalent to a supposedly inferior separate power-law representation. This is an odd claim given the algebraic (and consequently, numerical) identity between the two results. In fact, it is false because the representation of the pathway in biochemical systems theory is not properly aggregated; aggregation would result in loss of information about the fluxes in the two branches; this is true for the representation of the consumption of X_1 in their analysis (as represented by the parameters β_1 and h_{11}), but the aggregation is nullified by the representation of the fluxes through X_2 and X_3, which causes β_1 and h_{11} to become dependent parameters of those describing the two separate fluxes.

A consequence of the existence of these dependent parameters in the biochemical systems theory representation not pursued to its conclusion by Savageau *et al.* (1987) is the relationship between the two analyses if the effects of a change in enzyme 4 or 5 is considered. In their representation, a change in the activity of enzyme 4 causes a proportional change in the value of α_2, but also some change in the dependent parameter β_1, so that the total sensitivity to a change in α_2 is given by:

$$S'(Y, \alpha_2) = S(Y, \alpha_2) + S(Y, \beta_1)\frac{\partial \ln \beta_1}{\partial \ln \alpha_2}$$

From Savageau *et al.* (1987) we have:

$$\frac{\partial \ln \beta_1}{\partial \ln \alpha_2} = \frac{J_2}{J_1}$$

Consequently:

$$C_4^Y = S(Y, \alpha_2) + \frac{J_2}{J_1} \cdot S(Y, \beta_1)$$

and similarly for C_5^Y. Thus there is not in general a one-to-one correspondence between control coefficients and sensitivities, even in a linear pathway, and as a result the expression of the relationship between the summation theorem of metabolic control analysis and its equivalent in biochemical systems theory has never been given completely correctly by Savageau and his group. More important, it shows that biochemical systems theory is not constructed so as to give easily the enzyme-centred control coefficients of metabolic control analysis.

REFERENCES

Cascante, M., Franco, R. & Canela, E. I. (1988) in *Contrôle du métabolisme cellulaire* (Mazat, J.-P. & Reder, C., eds.), pp. 53-63, Bordeaux
Cornish-Bowden, A. (1989) *J. Theor. Biol. 136,* 365-377
Crabtree, B. & Newsholme, E. A. (1987) *Biochem. J. 247,* 113-120
Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561
Fell, D. A. & Snell, K. (1988) *Biochem. J. 256,* 97-101
Giersch, C. (1988*a*) *Eur. J. Biochem. 174,* 509-513
Giersch, C. (1988*b*) *Eur. J. Biochem. 174,* 515-519
Giersch, C. (1988*c*) *J. Theoret. Biol, 134,* 451-462
Hearn, A. C. (1985) *Reduce User's Manual,* Rand Corporation, Santa Monica, California
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95
Hofmeyr, J.-H. S. (1986*a*) Ph.D. Thesis, University of Stellenbosch
Hofmeyr, J.-H. S. (1986*b*) *Comp. Appl. Biosci. 2,* 5-11
Kacser, H. (1983) *Biochem. Soc. Trans. 11,* 35-40
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
LaBaume, L. B., Merrill, D. K., Clary, G. L. & Guynn, R. W. (1987) *Arch. Biochem. Biophys. 256,* 569-577
Reder, C. (1986) *Mimodrame mathématique sur les systèmes biochimiques,* Report No. 8608. UER de Mathématiques et d'Informatique, Université de Bordeaux I
Reder C. (1988) *J. Theor. Biol. 135,* 175-202
Reder, C. & Mazat, J-P. (1988) in *Contrôle du métabolisme cellulaire* (Mazat, J.-P. & Reder, C., eds.), pp. 77-96, Bordeaux
Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221
Savageau, M. A., Voit, E. O. & Irvine, D. H. (1987) *Math. Biosci. 86,* 147-169
Small, J. R. & Fell, D. A. (1989) *J. Theor. Biol. 136,* 181-197
Voit, E. O. & Savageau, M. A. (1987) *Biochemistry 26,* 6869-6880
Westerhoff, H. V. & Chen, Y.-D. (1984) *Eur. J. Biochem. 142,* 425-430
Westerhoff, H. V. & Kell, D. B. (1987) *Biotechnol. Bioeng. 30,* 101-107

Chapter 10

Performance Indices in Metabolic Systems: a Criterion for Evaluating Effectiveness in Metabolic Regulation

MARTA CASCANTE, RAFAEL FRANCO and ENRIC I. CANELA

A METABOLIC pathway is a dynamic system consisting of a set of level variables (concentrations of internal metabolites) with associated input and output fluxes (enzymic rates, and transport across membrane or combination of them). Furthermore, there must be at least one source metabolite or first metabolite and at least one final product. The pathway occurs in a viscous medium, sometimes heterogeneous with different diffusion rates in likely spatially organized clusters. Moreover, there are signals which, without matter transfer, communicate information from one point of the pathway to another. These signals make metabolic pathways self-regulated and are designed to give response to microenvironment changes of source metabolite or final product concentrations.

At present, there is increasing interest in analysing metabolic pathways as a whole, considering as fundamental aspects their quantitative evaluation and the optimality of the different regulatory mechanisms. Several approaches have been devised for these purposes (Savageau, 1969, 1976, 1987; Kacser & Burns, 1973; Heinrich & Rapoport, 1974, 1975; Crabtree & Newsholme, 1978, 1985, 1987; Westerhoff & Chen, 1984; Fell & Sauro, 1985; Sauro *et al.*, 1987; Voit & Savageau, 1987; Cascante *et al.*, 1989*ab*). On the other hand, quantitative analysis and optimality of self-regulated or externally regulated systems have been studied in many fields of science and technology by means of control theory (Barnet & Cameron, 1985) and sensitivity analysis (Tomovic & Vukobratovic, 1972; Frank, 1978; Rabitz *et al.*, 1983). In general, control theory and sensitivity analysis have been used to deal with linear systems, but a metabolic pathway consists of a network of enzyme reactions following non-linear rate equations, and, consequently, much of this information cannot be directly applied to this kind of systems. Therefore, to be operative, control theory and sensitivity analysis could be adapted to the characteristics of metabolic systems, introducing new definitions only when strictly necessary, and avoiding the redefinition of old concepts.

Marta Cascante, Rafael Franco and Enric I. Canela ● Departament de Bioquímica i Fisiología, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Catalunya, Spain

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

A key aspect in studying a metabolic pathway as a whole is to know how a perturbation in a parameter (i.e. any component of the system other than an internal metabolite: for instance, concentrations of final products, source substrates, enzymes, etc.) affects the response of the complete system. For this purpose an effectiveness criterion should be used. Control theory and sensitivity analysis employ different performance indices, although they are not necessarily valid for biochemical systems as performance indices are defined according to the interests of the system user. The aim of this work is to propound an *a priori* performance index adequate to be used in classical approaches used for analysing metabolic systems.

Theory

A performance index is a scalar (H) that provides a measure by which the performance of the system can be judged. Performance indices are in general arbitrary and depend on the interests of the user. For perturbations of an initial steady state SS_0 at time t_0 leading to another final steady state SS_1 at time t_1 the performance index can be defined in various ways [see Tomovic & Vukobratovic (1972), Frank (1978), Rabitz *et al.* (1983) for details], for example:

Minimum time: The system changes from the initial to the final state in the shortest possible time, and

$$H = t_1 - t_0 \tag{1}$$

Terminal control: The final steady state is as near as possible to the previous steady state:

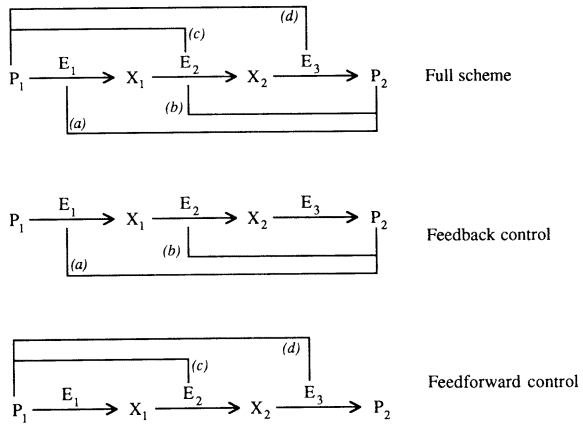
$$H = [\mathbf{X}(t_1) - \mathbf{X}(t_0)]^{\mathrm{T}}[\mathbf{M}][\mathbf{X}(t_1) - \mathbf{X}(t_0)] \tag{2}$$

where $\mathbf{X}(t_1)$ and $\mathbf{X}(t_0)$ represent the concentrations at final and initial steady states respectively. $[\mathbf{M}]$ could be the identity matrix; however, other values for some element would be used; in this case the corresponding internal metabolite would be more or less weighted using some subjective criterion.

Optimal track: The aim of this index is to measure the closeness of the trajectories followed by the metabolites to the initial steady state through the interval $t_0 \leq t \leq t_1$:

$$H = \int_{t_0}^{t_1} [\mathbf{X}(t_1) - \mathbf{X}(t_0)]^{\mathrm{T}}[\mathbf{M}][\mathbf{X}(t_1) - \mathbf{X}(t_0)]\mathrm{d}t \tag{3}$$

The criteria defined for control theory and sensitivity analysis can only be employed after the final steady state is reached, as the indices can only be calculated at t_1. Therefore,



Scheme 1. Unbranched pathway illustrating feedback and feedforward effects. The full scheme allows two feedback effects of P_2 on (*a*) E_1 and (*b*) E_2, and two feedforward effects of P_1 on (*c*) E_2 and (*b*) E_3. The two lower panels show schemes with feedback only (*ab*) or feedforward only (*cd*).

they are not suitable as *a priori* criteria, especially taking into account that metabolic equations are non-linear. For small perturbations, with a final steady state very close to the initial one, a performance index with respect to an infinitesimal change in a parameter Θ can be defined *a priori* according to the following formula:

$$H = [\mathbf{R}]^T[\mathbf{M}][\mathbf{R}] \tag{4}$$

where $[\mathbf{R}]^T$ is the vector $[R_1 \ldots R_i \ldots R_n]$, $R_i = d\ln x_i/d\ln\Theta$ and $[\mathbf{M}]$ is a real symmetric positive semi-definite weighting matrix. This performance index would be a measure of the resistance to change of the concentrations of internal metabolites. However, for metabolic systems other considerations would be taken into account.

Results

The system of an unbranched chain illustrated in Scheme 1 is completely defined by the following equations:

$$\dot{X}_1 = v_1(P_1, P_2, X_1, \Theta_1) - v_2(P_1, P_2, X_1, X_2, \Theta_2) \tag{5}$$

$$\dot{X}_2 = v_2(P_1, P_2, X_1, X_2, \Theta_2) - v_3(P_1, P_2, X_2, \Theta_3) \tag{6}$$

where v_i is the rate of the reaction catalysed by E_i. Under steady-state conditions $v_1 = v_2 = v_3 = J$, the flux for the system, and $\dot{X}_1 = \dot{X}_2 = 0$.

We can speak of perturbations when we change one of the external metabolites of the system, P_1 or P_2. Then we can use the relative sensitivity coefficients as a quantitative measure of the effect of a perturbation:

$$R_{P_j}^{X_i} = \frac{d\ln X_i}{d\ln P_j} \tag{7}$$

The matrix of relative sensitivity coefficients can be written as follows (Cascante *et al.*, 1989*a*):

$$[\mathbf{R}] = [\varepsilon]^{-1} \cdot [{}^{\kappa}\varepsilon] \tag{8}$$

which expresses the relationship between the following matrices. The matrix of response coefficients, [**R**] is as follows:

$$[\mathbf{R}] = \begin{bmatrix} R_1^J & R_2^J \\ R_1^{X_1} & R_2^{X_1} \\ R_1^{X_2} & R_2^{X_2} \end{bmatrix} \tag{9}$$

The matrix of elasticities, defined by $\varepsilon_j^i = \dfrac{\mathrm{d}\ln v_i}{\mathrm{d}\ln X_j}$, is as follows:

$$[\varepsilon] = \begin{bmatrix} 1 & -\varepsilon_1^1 & 0 \\ 1 & -\varepsilon_1^2 & -\varepsilon_2^2 \\ 1 & 0 & -\varepsilon_2^3 \end{bmatrix} \tag{10}$$

which has inverse as follows:

$$[\varepsilon]^{-1} = \frac{1}{D} \begin{bmatrix} \varepsilon_1^2\varepsilon_2^3 & -\varepsilon_1^1\varepsilon_2^3 & \varepsilon_1^1\varepsilon_2^2 \\ \varepsilon_1^3 - \varepsilon_2^2 & -\varepsilon_2^3 & \varepsilon_2^2 \\ \varepsilon_1^2 & -\varepsilon_1^1 & -\varepsilon_1^2 + \varepsilon_1^1 \end{bmatrix} \tag{11}$$

where $D = \varepsilon_1^2\varepsilon_2^3 - \varepsilon_1^1\varepsilon_2^3 + \varepsilon_1^1\varepsilon_2^2$ is the determinant of matrix [ε]. Finally, the matrix of special elasticities, defined by ${}^{\kappa}\varepsilon_j^i = \mathrm{d}\ln v_i/\mathrm{d}\ln P_j$, is as follows:

$$\left[{}^{\kappa}\varepsilon_j^i\right] = \begin{bmatrix} {}^{\kappa}\varepsilon_1^1 & {}^{\kappa}\varepsilon_2^1 \\ {}^{\kappa}\varepsilon_1^2 & {}^{\kappa}\varepsilon_2^2 \\ {}^{\kappa}\varepsilon_1^3 & {}^{\kappa}\varepsilon_2^3 \end{bmatrix} \tag{12}$$

For this system we can define the following performance indices (see Theory):

$$H_{P_i}^X = \begin{bmatrix} R_{P_i}^{X_1} & R_{P_i}^{X_1} \end{bmatrix} \cdot \begin{bmatrix} R_{P_i}^{X_1} \\ R_{P_i}^{X_2} \end{bmatrix} \tag{13}$$

Considering equivalent those systems having the same flux, matrix [ε] and identical constants ${}^{\kappa}\varepsilon_1^1 > 0$ and ${}^{\kappa}\varepsilon_2^3 < 0$, we have a set of equivalent systems differing in the values of the other terms of the special elasticity matrix. Using simple algebra we have found that the system with the lowest performance index among this set is that with all of the special elasticities for P_1 equal, i.e. P_1 affects the three enzymes equally, and the same for P_2.

In general, systems *in vivo* are simpler, and so many combined feedback and feed-

forward effects do not coexist. The above general pathway can be divided into the more restricted cases of feedback and feedforward control, illustrated in the lower two panels of Scheme 1. In each of these cases we have calculated the corresponding performance index and the appropriate special elasticity that minimizes this index, with the following results:

Case (a): Feedback control of E_1 *by* P_2:

$$H_{P_2}^X = \begin{bmatrix} R_{P_2}^{X_1} & R_{P_2}^{X_1} \end{bmatrix} \cdot \begin{bmatrix} R_{P_2}^{X_1} \\ R_{P_2}^{X_2} \end{bmatrix} \tag{14}$$

$$\kappa\varepsilon_2^1 = \frac{\kappa\varepsilon_2^3\left[\varepsilon_2^2\left(\varepsilon_2^3 - \varepsilon_2^2\right) + \left(-\varepsilon_1^2 + \varepsilon_1^1\right)\varepsilon_1^2\right]}{\left(\varepsilon_2^3 - \varepsilon_2^2\right)^2 + \left(\varepsilon_1^2\right)^2} \tag{15}$$

Case (b): Feedback control of E_2 *by* P_2:

$$H_{P_2}^X = \begin{bmatrix} R_{P_2}^{X_1} & R_{P_2}^{X_1} \end{bmatrix} \cdot \begin{bmatrix} R_{P_2}^{X_1} \\ R_{P_2}^{X_2} \end{bmatrix} \tag{16}$$

$$\kappa\varepsilon_2^2 = -\frac{\kappa\varepsilon_2^3\left[\left(\varepsilon_2^2\right)\left(-\varepsilon_2^3\right) + \left(-\varepsilon_1^2 + \varepsilon_1^1\right)\left(-\varepsilon_1^1\right)\right]}{\left(-\varepsilon_2^3\right)^2 + \left(-\varepsilon_1^1\right)^2} \tag{17}$$

Case (c): Feedforward control of E_2 *by* P_1:

$$H_{P_1}^X = \begin{bmatrix} R_{P_1}^{X_1} & R_{P_1}^{X_1} \end{bmatrix} \cdot \begin{bmatrix} R_{P_1}^{X_1} \\ R_{P_1}^{X_2} \end{bmatrix} \tag{18}$$

$$\kappa\varepsilon_1^2 = -\frac{\kappa\varepsilon_1^1\left[-\varepsilon_2^3\left(\varepsilon_2^3 - \varepsilon_2^2\right) + \left(\varepsilon_1^2\right)\left(-\varepsilon_1^1\right)\right]}{\left(-\varepsilon_2^3\right)^2 + \left(-\varepsilon_1^1\right)^2} \tag{19}$$

Case (d): Feedforward control of E_2 *by* P_1:

$$H_{P_1}^X = \begin{bmatrix} R_{P_1}^{X_1} & R_{P_1}^{X_1} \end{bmatrix} \cdot \begin{bmatrix} R_{P_1}^{X_1} \\ R_{P_1}^{X_2} \end{bmatrix} \tag{20}$$

$$\kappa\varepsilon_1^3 = \frac{\kappa\varepsilon_1^1\left[\varepsilon_2^2\left(\varepsilon_2^3 - \varepsilon_2^2\right) + \left(-\varepsilon_1^2 + \varepsilon_1^1\right)\varepsilon_1^2\right]}{\left(\varepsilon_2^2\right)^2 + \left(-\varepsilon_1^2 + \varepsilon_1^1\right)^2} \tag{21}$$

In real systems the sets of special elasticities are not in general optimal for minimizing these performance indices. For example, we have numerically evaluated performance indices in some simulated cases, selecting the feedback control cases (*a*, *b* and *a+b*), and assuming that enzymes follow the Michaelis-Menten equation with competitive product inhibition:

Table 1. Parameter Values and Performance Indices for an Unbranched Pathway*

	Enzyme 1				Enzyme 2				Enzyme 3			X_1 (mM)	X_2 (mM)	J (mol/L)	H_1	H_2	H_3
	V	K_m	K_i	K_{P_2}	V	K_m	K_i	K_{P_2}	V	K_m	K_i						
No inhibition	1	1	2		1.35	1	1.5		1.11	1	1	0.8067	1.227	0.4161	0.234	56	0.00077
Case (*a*)	1	0.5	1	1	1.35	1	1.5		1.11	1	1	0.7935	1.213	0.4129	0.072	48	0.00021
Case (*b*)	1	1	2		1.35	0.5	0.75	1	1.11	1	1	0.8148	1.224	0.4154	0.296	52	0.00079
Case (*a* + *b*)	1	0.5	0.75	1	1.35	0.5	0.75	1	1.11	1	1	0.8016	1.209	0.4122	0.026	47	0.00008

*The parameter values apply to the model shown in Scheme 1, and the performance indices are defined in the text. The values of H_2 and H_3 were obtained by perturbing the value of P_2 upward by 5%. In the original steady state the concentrations were X_1 = 0.8001 mM, X_2 = 1.200 mM and the flux was J = 0.4166 mol/L.

$$v_i = \frac{V_i X_{i-1}}{K_{mi}(1 + X_i/K_{i_i}) + X_{i-1}} \tag{22}$$

In cases (*a*, *b* and *a*+*b*) the competitive inhibition term includes the ratio P_2/K_{P_2} in the corresponding rate equation. The values for the parameters are shown in Table 1, and the concentrations are P_1 = 1 mM, X_1 = 0.8 mM, X_2 = 1.2 mM, P_2 = 1 mM. These constants and metabolite concentrations lead to an identical matrix [ε] in all three cases (*a*, *b* and *a*+*b*), and the flux is always the same. The matrix [ε] is as follows:

$$[\varepsilon] = \begin{bmatrix} 1 & 0.166 & 0 \\ 1 & -0.692 & 0.308 \\ 1 & 0 & -0.625 \end{bmatrix} \tag{23}$$

and the matrix of special elasticities for the control case is as follows:

$$[^{\kappa}\varepsilon] = \begin{bmatrix} 0.583 & 0 \\ 0 & -0.192 \\ 0 & -0.3125 \end{bmatrix}_a, \begin{bmatrix} 0.583 & -0.208 \\ 0 & 0 \\ 0 & -0.3125 \end{bmatrix}_b \text{ or } \begin{bmatrix} 0.583 & -0.208 \\ 0 & -0.192 \\ 0 & -0.3125 \end{bmatrix}_{a+b} \tag{24}$$

where the subscripts *a*, *b* and *a*+*b* indicate the cases of feedback inhibition referred to.

In order to evaluate the utility of the proposed performance index H_1, it was calculated in all of the above cases and compared with the minimum-time performance index H_2 and the terminal-control performance index H_3 (with [**M**] taken as the identity matrix). The two last ones were calculated by perturbing the final metabolite P_2 by 5% and applying eqns. (1-2) after the steady state was reached. The results are shown in the right-hand part of Table 1.

Discussion

The performance index defined by us in this work is easily applicable to any system in an *a priori* form. This index is quadratic, and provides a useful tool for weighting the peaks in

metabolite concentration that occur in the transition between steady states. This index, in the examples analysed, has a behaviour similar to that of terminal-control index. Probably this analogy would be lost if the system were unstable or if the variation of [**R**] with respect to the perturbation were important. It should be noted that [**R**] is not a constant, but depends on the metabolite concentrations. A controversial point of view with respect to the proposed performance index is the fact that in our approach the degree of flux modification is neglected.

It should be stressed that if the performance indices of a metabolic pathway are defined suitably they could be an excellent measure of the regulatory properties of the system. If by regulating we mean "buffering", i.e., to give minimal responses to modifications in external parameters, a well regulated system will be one whose internal metabolite concentrations are most insensitive to parameter modifications. This implies low values of [**R**] but also no sharp temporal peaks, i.e. accumulation of neither internal metabolites nor external effectors. The index defined here can be applied to all metabolic pathways. Between the simplest sequence having the minimum number of enzymic steps and a living organism there are many possible system models. A practical solution is to consider systems having as source metabolites or external products those that we wish to perturb. In this way these products will be considered as system parameters. Then, a more homogeneous treatment would be achieved. In general, this procedure presents no problems from a theoretical point of view as the number of steps can be reduced or increased according to the researcher's requirements. A different case would be the laboratory manipulation of the system, where there are experimental limitations. Furthermore, we should not lose sight of the fact that in living organisms only a relatively few products are external metabolites, and small systems are only laboratory simplifications. If we define the system so that the metabolite we would like to modify is an external effector, the regulatory properties should be defined considering that (*i*) flux is important and internal metabolite concentrations are of minor importance; or (*ii*) internal metabolite concentrations are of maximum importance and they should have minimum change. Then performance indices should involve both ideas. Generally, analysis of efficiency of regulatory systems is carried out focussing the interest on the performance for constant output; i.e. considering how the enzyme feedback system can maintain constant the value of the concentration of its final product when the concentrations of external metabolites change. The importance of performance indices becomes clear in contemplating the idea that feedback control is not necessarily responsible for homeostatic control, and that many constraint conditions are required for the system to have specific regulatory features.

It is interesting to consider a living organism as a system having a set of performance-indices for a set of source metabolites. Two different organisms of the same species could exist but with different internal metabolite concentrations because of having different parameters (e.g. enzyme concentrations or kinetic properties). Both organisms would have similar properties (internal metabolites and fluxes) but the index H would be different. The organism having the higher value of H could be worse regulated and could be less resistant to environmental changes; it would fail under some extreme conditions and would not survive. Accordingly, the H values would be a good measure of resistance to conditions of stress.

REFERENCES

Barnet, S. & Cameron, G. (1985) *Introduction to Mathematical Control Theory,* Clarendon Press, Oxford
Cascante, M., Franco, R. & Canela, E. I. (1989*a*) *Math. Biosci. 94,* 271-288
Cascante, M., Franco, R. & Canela, E. I. (1989*b*) *Math. Biosci. 94,* 289-309
Crabtree, B. & Newsholme, E. A. (1978) *Eur. J. Biochem. 89,* 19-22
Crabtree, B. & Newsholme, E. A. (1985) *Curr. Top. Cell. Reg. 25,* 21-76
Crabtree, B. & Newsholme, E. A. (1987) *Biochem. J. 247,* 113-120
Fell, D. A. & Sauro, H. M. (1985). *Eur. J. Biochem. 148,* 555-5619
Frank, P. M. (1978) *Introduction to System Sensitivity Theory,* Academic Press, New York
Heinrich, R. & Rapoport, S. M. (1974) *Eur. J. Biochem. 42,* 89-957
Heinrich, R. & Rapoport, S. M. (1975) *BioSystems 7,* 130-1368
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Rabitz, H. K., Kramer, M. & Dacol, D.(1983) *Ann. Rev. Phys. Chem. 34,* 1419-1461
Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221
Savageau, M. A. (1976) *J. Theor. Biol. 25,* 365-369
Savageau, M. A. (1976) *Biochemical System Analysis: A Study of Function and Design in Molecular Biology,* Addison-Wesley, Reading, Massachusetts
Savageau, M. A. (1987) *Trends Biochem. Sci. 12,* 219-2204
Tomovic, R. & Vukobratovic, M. (1972) *General Sensitivity Theory,* Elsevier, New York
Voit, E. O. & Savageau (1987) *Biochemistry 26,* 6869-6880
Westerhoff, H. V. & Chen, Y.-D. (1984) *Eur. J. Biochem. 142,* 425-430

Chapter 11

Practical Determination of Control Coefficients in Metabolic Pathways

ENRIC I. CANELA, MARTA CASCANTE
and RAFAEL FRANCO

IT IS OBVIOUS, not only for people working in metabolic control, that theory and practice can share common objectives but rarely share common roads, and it is often very difficult to put together in the same road theorists and experimentalists. In our opinion, metabolic control theories have also illustrated the difficulties in putting together these two disciplines: the hard nomenclature given by some control theories that is not easy for "test-tube" workers to follow; and the difficulties in directly measuring fluxes, intermediate concentrations or elasticity coefficients — terms introduced and popularized by metabolic control theory. In a given metabolic pathway, control of flux is exerted by the enzymes of the system. The direct calculation of flux sensitivities $\partial \ln J / \partial \ln E_i$, where J denotes a particular flux and E_i the concentration of an enzyme, implies calculation of flux before and after a small addition of enzyme.This is often extremely difficult to achieve. With this regard indirect methods exist by which local properties (elasticity or special elasticity coefficients) are calculated first. Then control coefficients are calculated by using the equations corresponding to the theorems of metabolic control theory.

Global and Local Properties

For a given system *global* properties are defined that depend on the way individual enzymatic steps are arranged together. In contrast, *local* properties are defined for each component (enzyme) of the system. The *flux control coefficients* and *concentration control coefficients* are are global properties defined, respectively, as follows:

$$C_{E_j}^{J} = \frac{\partial \ln J}{\partial \ln E_j}; \quad C_{E_j}^{S_i} = \frac{\partial \ln S_i}{\partial \ln E_j}$$

Enric I. Canela, Marta Cascante and Rafael Franco ● Departament de Bioquímica i Fisiologfa, Universitat de Barcelona, Martf i Franquès 1, 08028 Barcelona, Catalunya, Spain

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

where J is the flux of the system, S_i the concentration of any internal metabolite and E_j the concentration of any enzyme. If we extend the approach the the effect of an external effector with concentration X_p we have the *response coefficients* for flux and concentrations, which are defined respectively as follows:

$$R^J_{X_p} = \frac{\partial \ln J}{\partial \ln X_p}; \quad C^{S_i}_{X_p} = \frac{\partial \ln S_i}{\partial \ln X_p}$$

The substrate and enzyme *elasticities* are local properties, defined respectively as follows:

$$\varepsilon^i_{S_j} = \frac{\partial \ln v_i}{\partial \ln S_j}; \quad \mathrm{E}\varepsilon^i_{E_j} = \frac{\partial \ln v_i}{\partial \ln E_j}$$

If we extend the approach to the effect of an external effector we have the *kappa elasticities,* which are defined as follows:

$$\kappa\varepsilon^i_{X_p} = \frac{\partial \ln v_i}{\partial \ln X_p}$$

For the experimental calculation of global properties the complete system has to be studied, but for the experimental calculation of local properties isolated enzymes can be used. Flux control coefficients are a measure of how one global property, flux, varies when one independent variable of the system (enzyme concentration) varies.When genetic manipulation is possible one can measure this relationship directly (see below). When such a study is not possible the calculation of the local properties for the enzymes of the system should be the alternative. Fortunately, some relationships between global and local properties have been encountered since metabolic control theory was enunciated, derived from the theorems of this theory. More concretely, the first relationships were derived from the *connectivity theorem:*

$$\sum_{i=1}^{n} C^J_{E_i} \varepsilon^{v_i}_{S_k} = 0$$

which constitutes a set of equations by which if local properties (elasticities) are known, some global properties can be easily calculated. The theorems of metabolic control theory were first expressed by Fell & Sauro (1985) in matrix terms by calculating the flux control coefficients of enzymes in a metabolic pathway from their elasticities. In subsequent studies, Sauro *et al.* (1987), Fell *et al* (1988) and Small & Fell (1989) extended the matrix algebra to include other global properties such as the concentration control coefficients. The development of matrix algebra relating local and global properties is valuable even for experimentalists. Henceforth they should calculate coefficients that are easier to obtain experimentally, irrespective of whether they constitute local or global properties. The remaining coefficients will be calculated using matrix algebra. The most feasible relation-

ship between elasticities and control coefficients is that given by Cascante *et al.* (1988, 1989*ab*). From a study of general sensitivity applied to metabolic processes we have developed a "logical" matrix algebra in an *a priori* form. The equation that represents this development[1] is simply given by

$$\begin{bmatrix}\text{Matrix of}\\ \text{global properties}\end{bmatrix} = \begin{bmatrix}\text{Matrix of local properties:}\\ \text{substrate elasticities}\end{bmatrix}^{-1} \cdot \begin{bmatrix}\text{Matrix of local properties:}\\ \text{enzyme elasticities}\end{bmatrix} \quad (1)$$

The matrix of global properties consists either of flux or of concentration control coefficients. The matrix of enzyme elasticities is simply the identity matrix when velocities are homogeneous functions with respect to enzyme concentrations ($v = kE$).When this occurs, it follows from eqn. (1) that the theorems of metabolic control theory are obtained in an *a posteriori* form. Since Fell and coworkers developed the algebra from the theorems, we think that it would be considered as *a posteriori* algebra. Our algebra summarized in eqn. (1) can be applied to any system. For a linear chain:

$$X_0 \xrightarrow{E_1} S_1 \ \ldots\ldots\ S_{n-1} \xrightarrow{E_n} X_p$$

and assuming that velocities are proportional to enzyme concentrations, eqn. (1) can be written explicitly as follows:

$$\begin{bmatrix} C_1^J & \cdots & C_n^J \\ C_1^{S_1} & \cdots & C_n^{S_1} \\ \vdots & \ddots & \vdots \\ C_n^{S_n} & \cdots & C_n^{S_n} \end{bmatrix} = \begin{bmatrix} 1 & -\varepsilon_1^1 & \cdots & -\varepsilon_n^1 \\ \vdots & \vdots & \ddots & \vdots \\ 1 & -\varepsilon_1^n & \cdots & -\varepsilon_n^n \end{bmatrix}^{-1} \quad (2)$$

Experimental Methods for Calculating Control Coefficients

We will address this chapter to the experimental calculation of control coefficients. However, we should not forget an indirect way to proceed by which mathematical models mimicking the kinetic properties of the systemic enzymes are built, which many successful studies leading to the determination of control coefficients in a variety of pathways have used, totally or partially (Rapoport *et al.*, 1974, 1976; Heinrich & Rapoport, 1983; Brumen & Heinrich, 1984; LaPorte *et al.*, 1984; Walsh & Koshland, 1984; Werner & Heinrich, 1985; Groen *et al.*, 1986, Sorribas & Bartrons, 1986; Canela *et al.*, 1987; Torres *et al.*, 1988*b*; Fell & Snell, 1988, Fell *et al.*, 1988).

Genetic handling. Control coefficients are easily obtained by direct construction of

[1]We are grateful to Dr H. Sauro for pointing out the existence of the enzyme-enzyme interactions problem at the meeting organized by J.-P. Mazat and C. Reder in Bordeaux in 1987 (see Mazat & Reder, 1988). As a consequence we realized that our algebra could deal with such a problem very easily.

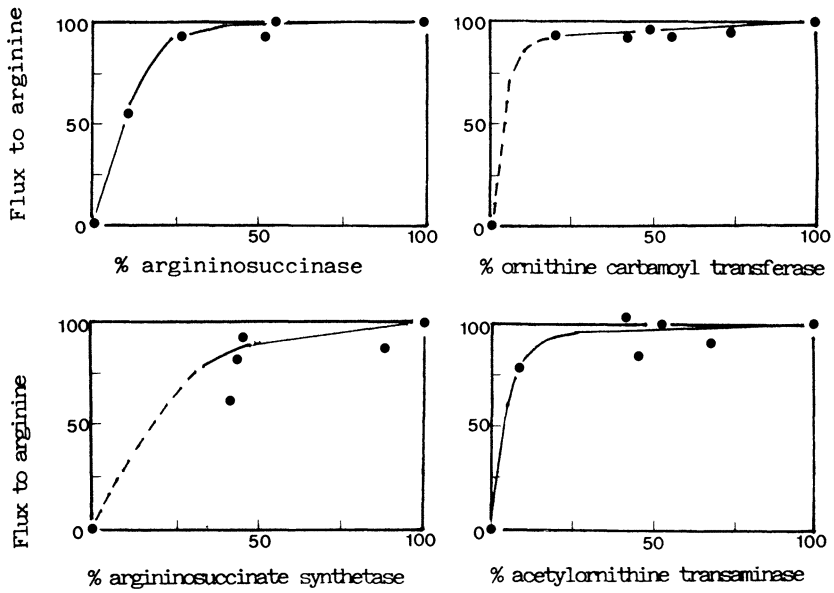


Figure 1. Flux variation with respect to enzyme concentration: a genetic approach using *Neurospora* heterokaryons. Redrawn from Kacser & Burns (1981) with permission.

plots of flux against enzyme activity (Fig. 1). To our knowledge this is the best way to do it. However, these kind of plots as that given by Kacser & Burns (1981) can be only obtained by genetic handling, i.e. by means of suitable mutants bearing different activity for an enzyme of the pathway studied (Flint *et al.*, 1980; Flint *et al.*, 1981, Middleton *et al.*, 1983; Stuart *et al.*, 1986). This procedure is feasible for microorganisms. In animal cells variations of enzyme activity can be achieved by modifying the metabolic states of the cell. Thus, Salter *et al.* (1986; see also Chapter 32 of this book) have quantified the flux control coefficients for the enzymes of aromatic amino acid metabolism in rat liver. In a complete study they induced different metabolic states by pretreating rats in various ways: adrenalectomy, starvation, chronic diabetes, etc. (Fig. 2).

Using genetic procedures one can be sure of which enzyme activity is changing. In contrast, by the method employed by Salter *et al.* (1986) one might expect that after a given treatment more than one enzyme might vary. The importance of this fact in bias in final results remains to be determined; depending upon the metabolism studied it can be minimal or it can have be relatively important.

Enzyme titration. This method developed recently by Meléndez-Hevia and coworkers has been applied to various metabolic systems (Torres *et al.*, 1986; Torres *et al.*, 1988) and is fully described in Chapter 18 of this book. It requires the external addition of the enzymes of the pathway. These enzymes can be supplied by companies or purified by biochemists. When this is possible the method can be very reliable. Unfortunately sometimes it is difficult to obtain isolated enzymes for the pathway of interest.

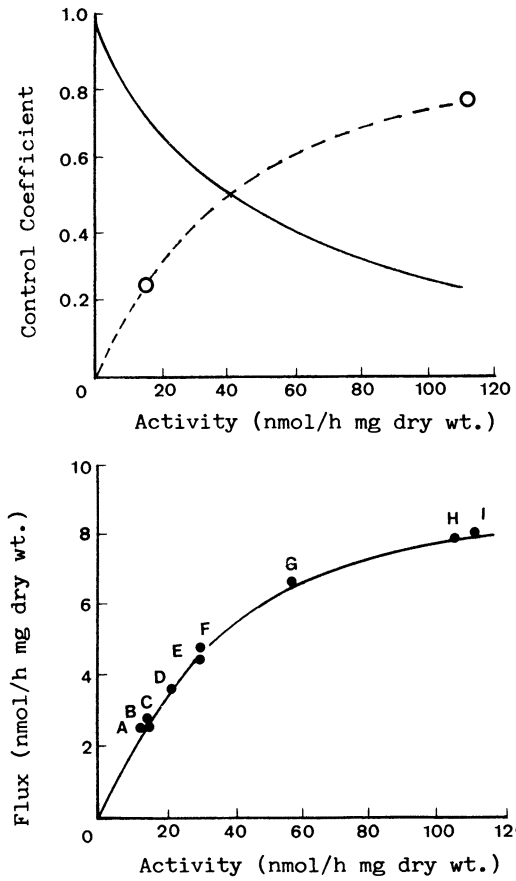


Figure 2. Flux variation with respect to tryptophan 2,3-dioxygenase activity: a metabolic approach in rat liver. The figure shows the flux through tryptophan 2,3-dioxygenase from cells pre-treated in various ways: A, adrenolectomized; B, control; C, starved for 24 hours; D, control (cells made at 16:30 h); E, held overnight in the laboratory; F, chronic diabetic plus insulin; G, chronic diabetic; H, pre-treated with dimethasone phosphate; I, separate cell preparations. Redrawn from Salter *et al.* (1986) with permission.

The combined response coefficients method. This is the method which has been by far the most used. Results with it for control in mitochondrial respiration (Groen *et al.*, 1982*ab*; Wanders *et al.*, 1984; Mazat *et al.*, 1986), citrulline synthesis (Wanders *et al.*, 1983; Wanders *et al.*, 1984*a*) and gluconeogenesis (Groen *et al.*, 1986) constitute a clear example of how experimentation can lead to the understanding of how control is exerted in complex pathways. For a given external effector, such as an inhibitor modulating the activity of E_i, the following relationship applies:

$$\frac{dJ}{J}\Big/\frac{dI}{I} = C_i\left(\frac{\delta v_i}{v_i}\Big/\frac{\delta I}{I}\right)$$

Thus, the control coefficient can be obtained by taking the limit at $I = 0$:

$$C_i^J = \left(\frac{\frac{dJ}{J}}{dI}\right) \Big/ \left(\frac{\frac{\delta v_i}{v_i}}{\delta I}\right)$$

The numerator of this expression can be determined from the initial slope of the flux inhibition curve and the denominator can be calculated by knowing the kinetics of the enzyme with respect to the inhibitor. Note that irreversible inhibitors, when possible, are preferable because then the following equation applies:

$$C_i^J = -\frac{dJ}{J} \Big/ \frac{dI}{I_{max}}$$

where I_{max} is the amount of inhibitor required for complete inhibition of the enzyme. Carboxyatractyloside, an irreversible inhibitor of the adenine nucleotide translocator, has been used in studies of the control of mitochondrial respiration (Groen *et al.*, 1982*ab*; Tager *et al.*, 1983; Wanders *et al.*, 1984*b*; Mazat *et al.*, 1986). Norvaline, a inhibitor of ornithine transcarbamoylase, competitive with respect to ornithine, has been used to study the control exerted by the enzymatic steps leading to the synthesis of citrulline in mitochondria (Wanders *et al.*, 1983; 1984*a*).

It should be emphasized again that this method has been used more than others requiring experimental effort. However for applying the method to the control study of a given pathway care must be taken with respect to the inhibitor used. Such an inhibitor must affect only one enzyme of the pathway, and it should pass cellular membranes freely; otherwise one cannot be sure of the actual concentration of the inhibitor, for example, inside mitochondria. Finally the inhibitor must not be metabolized within the cell. All of this makes it extremely difficult to obtain suitable inhibitors of the kind needed for control studies.

Indirect calculation of elasticities. As mentioned above, elasticities can be determined from the rate laws of the enzymes. For experimental calculation of elasticities, Kacser & Burns (1979) proposed a method by which two steady states are considered, differing, for instance, in the flux through the first step catalysed by E_1. For the two steady states *a* and *b* we have

$$\frac{dJ_a}{J_a} = \varepsilon_{S_1}^2 \frac{dS_{1a}}{S_{1a}} + \varepsilon_{S_1}^2 \frac{dS_{2a}}{S_{2a}}$$

$$\frac{dJ_b}{J_b} = \varepsilon_{S_1}^2 \frac{dS_{1b}}{S_{1b}} + \varepsilon_{S_1}^2 \frac{dS_{2b}}{S_{2b}}$$

Thus, if we are able to measure the variations of the flux J and of the concentrations S_1 and S_2, we have a set of two equations in two unknown elasticities. Combining this method with the use of specific inhibitors Wanders *et al.* (1983) calculated the control of the steps of citrulline synthesis. Groen *et al.* (1986) used this method with others (one more direct and

one more theoretical) for calculating elasticities when studying the control of gluconeogenesis in rat liver cells. 3-Mercaptopicolinic acid has been used to calculate the elasticity of oxaloacetate transport across the mitochondrial membrane (Groen *et al.*, 1986).

Direct calculation of elasticities. Direct calculation of elasticity coefficients is very simple with the isolated enzymes. But the problem is to test the enzyme *in vitro* but in the appropriate conditions, which are those *in vivo*. It is preferable to obtain elasticities by leaving the enzymes within the pathway, but this is very difficult to achieve.

A direct calculation of the elasticity coefficient of pyruvate kinase with respect to phosphoenolpyruvate was reported by Groen *et al.* (1986). They assumed that only the concentration of phosphoenolpyruvate changes when the cytosolic redox state varies in presence of glucagon. Also, inhibitors can be used to modify an enzyme activity and thus calculating elasticities if the concentration of the intermediate can be measured in the different steady states (with and without the inhibitor). Norvaline and malonate have been used by Wanders *et al.* (1983, 1984*a*) to calculate, respectively, elasticity of carbamoylphosphate synthetase and of ornithine transcarbamoylase with respect to carbamoyl phosphate. In this chapter we provide a method for direct calculation of elasticity coefficients.

Methods

Metabolism. The pathway studied has been that leading to uric acid from purine bases (Fig. 3). Steps 1 and 3 are catalysed by xanthine oxidase (EC 1.1.3.22) and step 2 by guanine aminohydrolase (EC 3.5.4.3). Application of the matrix algebra of Cascante *et al.* (1989*ab*) to this pathway lead to the following relationships:

$$\begin{bmatrix} J_2/J_1 & J_3/J_1 & -\varepsilon^1_{\text{xanthine}} \\ 1 & 0 & -\varepsilon^2_{\text{xanthine}} \\ 0 & 1 & -\varepsilon^3_{\text{xanthine}} \end{bmatrix} \cdot \begin{bmatrix} C^{J_2}_{\text{x oxidase}} & C^{J_2}_{\text{g aminohydrolase}} \\ C^{J_3}_{\text{x oxidase}} & C^{J_3}_{\text{g aminohydrolase}} \\ C^{\text{xanthine}}_{\text{x oxidase}} & C^{\text{xanthine}}_{\text{g aminohydrolase}} \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ 0 & 1 \\ 1 & 0 \end{bmatrix} \qquad (3)$$

$$[\varepsilon] \qquad [C] \qquad [H]$$

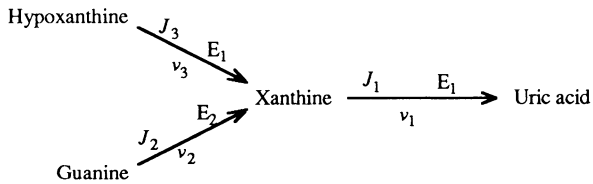


Figure 3. Scheme of the final part of purine catabolism. Enzymes: reactions 1 and 3, xanthine oxidase (EC 1.1.3.22); reaction 2, guanine aminohydrolase (EC 3.5.4.3). The rate equations for the three enzymes as used in our theoretical model were as follows: $v_1 = 4.0 \times 10^{-7}[\text{xanthine}]^2/(1.2 \times 10^{-10} + 1.1[\text{xanthine}]^2 + 0.9[\text{hypoxanthine}]^2\ 2.0 \times 10^{-5}[\text{uric acid}])$; $v_2 = 1.8 \times 10^{-7}[\text{guanine}]/(5.0 \times 10^{-5} + [\text{guanine}] + 0.5[\text{xanthine}])$; $v_3 = 4.0 \times 10^{-7}[\text{hypoxanthine}]^2/(1.2 \times 10^{-10} + 1.1[\text{xanthine}]^2 + 0.9[\text{hypoxanthine}]^2\ 2.0 \times 10^{-5}[\text{uric acid}])$.

$$[C] = [\varepsilon]^{-1}[H] \tag{4}$$

$$C^{J_1}_{\text{x oxidase}} = \left(\frac{J_2}{J_1}\right) C^{J_2}_{\text{x oxidase}} + \left(\frac{J_3}{J_1}\right) C^{J_3}_{\text{x oxidase}} \tag{5}$$

$$C^{J_1}_{\text{g aminohydrolase}} = \left(\frac{J_2}{J_1}\right) C^{J_2}_{\text{g aminohydrolase}} + \left(\frac{J_3}{J_1}\right) C^{J_3}_{\text{g aminohydrolase}} \tag{6}$$

$$\begin{bmatrix} J_2/J_1 & J_3/J_1 & -\varepsilon^1_{\text{xanthine}} \\ 1 & 0 & -\varepsilon^2_{\text{xanthine}} \\ 0 & 1 & -\varepsilon^3_{\text{xanthine}} \end{bmatrix} \cdot \begin{bmatrix} R^{J_2}_{\text{hypoxanthine}} & R^{J_2}_{\text{guanine}} & R^{J_2}_{\text{uric acid}} \\ R^{J_3}_{\text{hypoxanthine}} & R^{J_3}_{\text{guanine}} & R^{J_3}_{\text{uric acid}} \\ R^{\text{xanthine}}_{\text{hypoxanthine}} & R^{\text{xanthine}}_{\text{guanine}} & R^{\text{xanthine}}_{\text{uric acid}} \end{bmatrix} = \begin{bmatrix} {}^{\kappa}\varepsilon^1_{\text{hypoxanthine}} & {}^{\kappa}\varepsilon^1_{\text{guanine}} & {}^{\kappa}\varepsilon^1_{\text{uric acid}} \\ {}^{\kappa}\varepsilon^2_{\text{hypoxanthine}} & {}^{\kappa}\varepsilon^2_{\text{guanine}} & {}^{\kappa}\varepsilon^2_{\text{uric acid}} \\ {}^{\kappa}\varepsilon^3_{\text{hypoxanthine}} & {}^{\kappa}\varepsilon^3_{\text{guanine}} & {}^{\kappa}\varepsilon^3_{\text{uric acid}} \end{bmatrix} \tag{7}$$

$[\varepsilon]$ $[R]$ $[{}^{\kappa}\varepsilon]$

$$[R] = [\varepsilon]^{-1}\,[{}^{\kappa}\varepsilon] \tag{8}$$

After experimental calculation of elasticities, flux and concentration control coefficients will be calculated by solving these equations.

Calculation of elasticities. Elasticities defined as above under *Local and Global Properties* were calculated before (v_1, v_2 and v_3) and after (v'_1, v'_2 and v'_3) the addition of a small amount of the corresponding intermediate. Thus, a given elasticity or special elasticity is calculated as follows:

$$\varepsilon^i_j = \left(\frac{v'_i - v_i}{\Delta S_j}\right)\left(\frac{S_j}{v_i}\right) \tag{9}$$

the intermediate (S_j) being guanine, hypoxanthine, xanthine or uric acid.

Measurement of velocities. Velocities were calculated by measuring at each time the exact concentrations of all the metabolites of the system. For this purpose absorbance at various wavelengths in the UV region was measured. We used a diode array 8450 A Hewlett-Packard spectrophotometer. Absorbance was measured at 1 nm intervals in the range 240-300 nm, and the spectra of hypoxanthine, xanthine, guanine and uric acid in this range are shown in Fig. 4. These spectra, corresponding to samples of known concentration, are stored as standards in the memory of the spectrophotometer. For a given mixture the multicomponent analysis subroutine of the apparatus calculates the actual concentration of each metabolite. The mathematical technique used is a weighted least-squares regression that produces a solution for as many simultaneous equations as there are data points within the wavelength range specified for the analysis. After performing such calculations at various times it is possible to obtain the velocities for each of the steps indicated in Fig. 3. In the case of non-linear relationships between concentration and time, a second degree polynomial function was adjusted by non-linear regression and the velocity was calculated by differentiation at

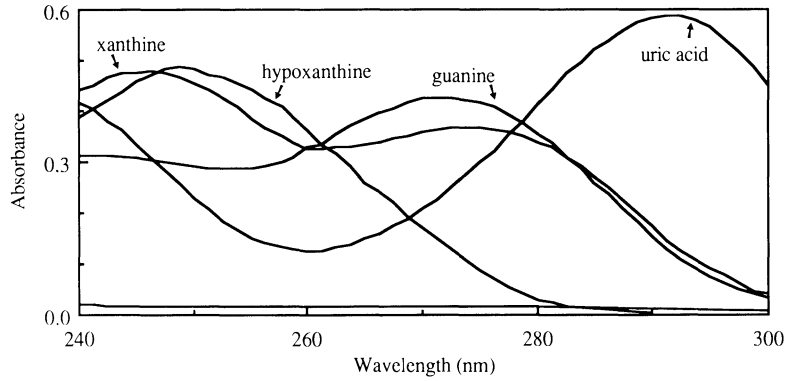


Figure 4. Spectra of xanthine, hypoxanthine, guanine and uric acid. The concentration of each compound was 50 μM.

zero time. The system, initially being in steady state, was constructed by adding suitable amounts of metabolites and of commercial enzymes: xanthine oxidase and guanine amino-hydrolase (Boehringer Mannheim). By the previous procedure it was verified that concentration of xanthine did not vary with time. In these control conditions v_1, v_2 and v_3 were calculated. Obviously, $v_2 + v_3 = v_1$.

Method of Simulation in a Theoretical Model. The dynamic behaviour of the pathway displayed in Fig. 3 has been simulated by using the equations obtained in our laboratory for isolated enzymes, and using a numerical method of resolution of differential equations (see Franco & Canela, 1984, and Canela *et al.*, 1986). The equations are given in the legend to Fig. 3. Simulated elasticities were calculated for xanthine oxidase and guanine amino-hydrolase as follows: the change in velocities through J_1, J_2 and J_3 has been calculated by simulating the dynamic behaviour after changing the concentration of the corresponding metabolite. Our experimental conditions (see below) have been used to perform this simulation. Elasticities were then calculated by applying eqn. (9).

Results

As a preliminary finding we should emphasize that if we are able to experimentally calculate the actual velocities through J_1, J_2 and J_3, after changing by 10% the corresponding metabolite concentration, errors in determining elasticities are negligible. Another question arises when we consider that the experimentally encountered velocity is not the true velocity at zero time.Then we have performed simulations in order to assess how great is the experimental error in determining elasticities by our method (see below).

Experimental system. Our experimental steady state had the following characteristics (Fig. 3): [xanthine] = 19.2 μM; flux through v_1 = 0.110 μmol/s; flux through v_2 = 0.038 μmol/s; flux through v3 = 0.072 μmol/s. At time zero the concentrations of external effectors

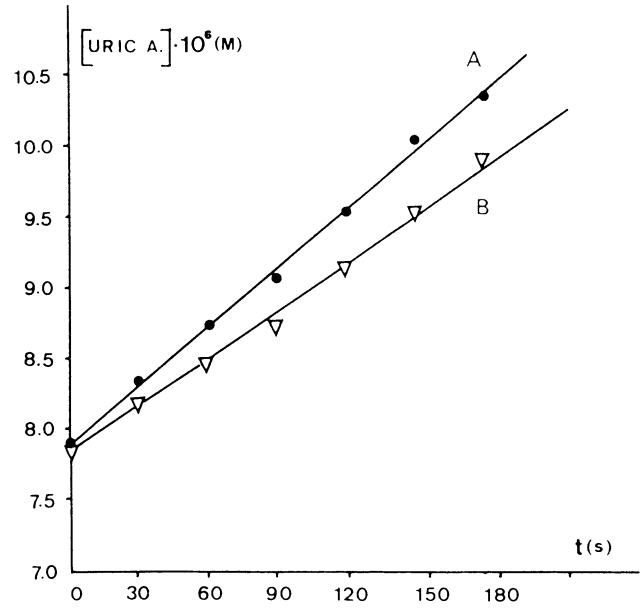


Figure 5. Graphical procedure for calculating elasticities: a practical example. The concentrations of xanthine were 19.2 μM (line A) and 15.3 μM (line B); the other concentrations were the same for both lines: [hypoxanthine] = 9.59 μM, [guanine] = 14.9 μM, [uric acid] = 7.68 μM. The rates were A, 13.2 ± 0.3 nM/s and B, 11.5 ± 0.4 nM/s, from which one may calculate the elasticity as [(13.2 - 11.5)/(19.2 - 15.3)](19.2/13.2) = 0.63.

were as follows: [hypoxanthine] = 9.6 μM; [guanine] = 14.9 μM; [uric acid] = 7.7 μM.

Elasticities with respect to xanthine were obtained by decreasing by 10% the xanthine concentration and measuring the new velocity in each reaction (v_1',v_2' and v_3'). The new concentration of xanthine was 17.3 μM at zero time. Similarly, special elasticities with respect to hypoxanthine, guanine and uric acid were obtained, by decreasing by 10% the concentration of the corresponding compound and measuring the new velocities in each reaction (v_1',v_2' and v_3'). The new concentrations at time zero for hypoxanthine, guanine and uric acid were respectively 8.6, 13.4 and 6.9 μM.

Elasticities. The three elasticities with respect to xanthine and the nine special elasticities with respect to external metabolites were then calculated by applying eqn. (9). In Fig. 5 a graphical procedure is indicated for calculating $\varepsilon^1_{\text{xanthine}}$. Results are indicated in Table 1, and are compared with those obtained by simulation. Though qualitatively similar, elasticities with respect to xanthine differ in magnitude between our experimental model and the theoretical one. In the real case it seems that xanthine has more influence over guanine aminohydrolase than expected by our theoretical rate law for this enzyme. The effect of xanthine over the two steps catalysed by xanthine oxidase is more difficult to explain. The special elasticities show the same sort of behaviour comparing experimental and theoretical models: qualitative agreement but disagreement in magnitude (Table 1). The high special elasticity of guanine aminohydrolase with respect to hypoxanthine, which theoretically should be zero, is remarkable. This indicates that the concentration of hypoxanthine should be included in the theoretical rate law for guanine aminohydrolase. Comparing the true elasticities and special elasticities of our theoretical model with those obtained by simulation (time = 20 arbitrary units) we found that "systematic experimental errors" are very low. The

Table 1. Comparison between experimentally determined and calculated control coefficients and elasticities for purine metabolism .

Algebraic expression	Experimental	Calculated from model	Simulated with model
$\begin{bmatrix} J_2/J_1 & J_3/J_1 & -\varepsilon^1_{\text{xanthine}} \\ 1 & 0 & -\varepsilon^2_{\text{xanthine}} \\ 0 & 1 & -\varepsilon^3_{\text{xanthine}} \end{bmatrix}$	$\begin{bmatrix} 0.340 & 0.660 & -0.630 \\ 1 & 0 & 0.710 \\ 0 & 1 & 1.940 \end{bmatrix}$	$\begin{bmatrix} 0.340 & 0.660 & -1.390 \\ 1 & 0 & 0.08357 \\ 0 & 1 & 0.606 \end{bmatrix}$	$\begin{bmatrix} 0.340 & 0.660 & -1 \\ 1 & 0 & 0.0600 \\ 0 & 1 & 0.433 \end{bmatrix}$
$\begin{bmatrix} {}^{\kappa}\varepsilon^1_{\text{hypoxanthine}} & {}^{\kappa}\varepsilon^1_{\text{guanine}} & {}^{\kappa}\varepsilon^1_{\text{uric acid}} \\ {}^{\kappa}\varepsilon^2_{\text{hypoxanthine}} & {}^{\kappa}\varepsilon^2_{\text{guanine}} & {}^{\kappa}\varepsilon^2_{\text{uric acid}} \\ {}^{\kappa}\varepsilon^3_{\text{hypoxanthine}} & {}^{\kappa}\varepsilon^3_{\text{guanine}} & {}^{\kappa}\varepsilon^3_{\text{uric acid}} \end{bmatrix}$	$\begin{bmatrix} -0.57 & 0.065 & -0.26 \\ -3.59 & 1.06 & 0.058 \\ 2.19 & -0.35 & -0.047 \end{bmatrix}$	$\begin{bmatrix} -0.326 & 0 & -0.298 \\ 0 & 0.790 & 0 \\ 1.67 & 0 & -0.298 \end{bmatrix}$	$\begin{bmatrix} -0.00483 & 0.0479 & -0.286 \\ -0.0187 & 0.803 & -0.0014 \\ 1.526 & -0.0266 & -0.3186 \end{bmatrix}$
$\begin{bmatrix} C^{J_1}_{\text{x oxidase}} & C^{J_1}_{\text{g aminohydrolase}} \\ C^{J_2}_{\text{x oxidase}} & C^{J_2}_{\text{g aminohydrolase}} \\ C^{J_3}_{\text{x oxidase}} & C^{J_3}_{\text{g aminohydrolase}} \\ C^{\text{xanthine}}_{\text{x oxidase}} & C^{\text{xanthine}}_{\text{g aminohydrolase}} \end{bmatrix}$	$\begin{bmatrix} 0.900 & 0.100 \\ 0.112 & 0.888 \\ 1.307 & -0.307 \\ -0.158 & 0.158 \end{bmatrix}$	$\begin{bmatrix} 0.738 & 0.262 \\ 0.0158 & 0.984 \\ 1.114 & -0.114 \\ -0.189 & 0.189 \end{bmatrix}$	$\begin{bmatrix} 0.737 & 0.263 \\ 0.0158 & 0.984 \\ 1.114 & -0.114 \\ -0.189 & 0.189 \end{bmatrix}$
$\begin{bmatrix} R^{J_2}_{\text{hypoxanthine}} & R^{J_2}_{\text{guanine}} & R^{J_2}_{\text{uric acid}} \\ R^{J_3}_{\text{hypoxanthine}} & R^{J_3}_{\text{guanine}} & R^{J_3}_{\text{uric acid}} \\ R^{\text{xanthine}}_{\text{hypoxanthine}} & R^{\text{xanthine}}_{\text{guanine}} & R^{\text{xanthine}}_{\text{uric acid}} \end{bmatrix}$	$\begin{bmatrix} -3.85 & 1.039 & 0.148 \\ 1.47 & -0.408 & 0.20 \\ 0.369 & 0.030 & -0.126 \end{bmatrix}$	$\begin{bmatrix} -0.0654 & 0.777 & -0.0047 \\ 1.1954 & -0.090 & -0.332 \\ 0.783 & 0.149 & 0.0563 \end{bmatrix}$	$\begin{bmatrix} -0.065 & 0.793 & 0.00489 \\ 1.194 & -0.0963 & -0.3439 \\ 0.767 & 0.161 & 0.0584 \end{bmatrix}$

only exception is the value for $^{\kappa}\varepsilon^{1}_{\text{hypoxanthine}}$, which changes from -0.326 (real value for the theoretical model) to -0.000483, and this can even be made positive by extending the time of simulation. This is explained by taking into consideration the accumulation of xanthine, which is also a substrate of xanthine oxidase, after perturbing the initial steady state by increasing the hypoxanthine concentration.

Control coefficients. Control coefficients were calculated from the elasticities by employing eqns. (3-6). Flux control coefficients for the main flux J_1 are, as expected from comparing the elasticity values, different for the experimental and theoretical models (Table 1). However, in both cases xanthine oxidase has far more control than guanine aminohydrolase. Also, as expected after inspecting the pathway, the flux control coefficient of flux J_3 with respect to xanthine oxidase is higher than one whereas that of J_3 with respect to guanine aminohydrolase is negative. Notwithstanding this, the sum of the two control coefficients is unity. The concentration control coefficient of xanthine with respect to xanthine oxidase is negative and that with respect to guanine aminohydrolase is positive. The values are similar for both the theoretical and the experimental models. With respect to the response control coefficients for fluxes and for xanthine, we have the same qualitative response in both theoretical and experimental models (Table 1). It should be noted that, as the values differ when comparing the theoretical model with the experimental model whereas the values for the simulated model are very close to the theoretical actual values, we can conclude that our experimentally obtained results are likely to be very close to reality.

From the results shown above, it is clear that the pathway studied has a high inertia to change either the flux control coefficients or the xanthine concentration control coefficients with respect to xanthine oxidase or guanine aminohydrolase. Thus, if the results of Table 1 are analysed carefully it is remarkable that even a relatively high change in the values of some elasticities does not lead to appreciable variation in these control coefficients.

REFERENCES

Brumen, M. & Heinrich, R. (1984) *BioSystems 17,* 155-169

Canela, E. I., Ginesta, I. & Franco, R. (1986) *Arch. Biochem. Biophys. 254,* 142-153

Cascante, M., Franco, R. & Canela, E. I. (1988) in *Contrôle du metabolisme cellulaire* (Mazat, J.-P. & Reder, C., eds.) pp. 53-63, Bordeaux

Cascante, M., Franco, R. & Canela, E. I. (1989*a*) *Math. Biosci. 94,* 271-288

Cascante, M., Franco, R. & Canela, E. I. (1989*b*) *Math. Biosci. 94,* 289-309

Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 155-161

Fell, D. A., Sauro, H. M. & Small, J. R. (1988) in *Contrôle du metabolisme cellulaire* (Mazat, J.-P. & Reder, C., eds.) pp. 15-26, Bordeaux

Fell, D. A. & Snell, K. (1988) *Biochem. J. 256,* 97-101.

Flint, H. J., Porteous, D. J. & Kacser, H. (1980) *Biochem. J 190,* 1-15

Flint, H. J., Tateson, R. W., Barthelmess, I. B., Porteous, D. J., Donachie, W. D. & Kacser, H. (1981) *Biochem. J. 200,* 155-161

Franco, R. & Canela, E. I. (1984) *Eur. J. Biochem. 144,* 305-315

Groen, A. K., Van der Meer, R., Westerhoff, H. V., Wanders R. J., Akerboom, T. P. M. & Tager, J. M. (1982*a*) in *Metabolic Compartmentation* (Sies, H., ed.) pp. 9-37, Academic Press, New York

Groen, A. K., Wanders R. J. A., Westerhoff, H. V., Van der Meer, R. & Tager, J. M. (1982*b*) *J. Biol. Chem. 257,* 2754-2757
Groen, A. K., van Roermund, C. W. T., Vervoorn, R. C. & Tager, J. M. (1986) *Biochem. J. 237,* 379-389
Heinrich, R. & Rapoport, S. M. (1983) *Biochem. Soc. Trans. 11,* 31-35
Kacser, H. & Burns, J. A. (1979) *Biochem. Soc. Trans. 7,* 1149-1160
Kacser, H. & Burns, J. A. (1981) *Genetics 97,* 639-666
LaPorte, D. C., Walsh, K. & Koshland, D. E., Jr. (1984) *J. Biol. Chem. 259,* 14068-14075
Mazat, J.-P., Jean-Bart, E., Rigoulet, M. & Guérin, B (1986) *Biochim. Biophys. Acta 849,* 7-15
Mazat, J.-P. & Reder, C. (eds.) (1988) *Contrôle du metabolisme cellulaire,* Bordeaux
Middleton, R. J. & Kacser, H. (1983) *Genetics 105,* 633-650
Rapoport, T. A., Heinrich, R., Jacobasch, G. & Rapoport, S. M. (1974) *Eur J Biochem 42,* 107-120
Rapoport, T. A. & Heinrich, R. (1975) *BioSystems 7,* 120-129
Rapoport, T. A., Heinrich, R. & Rapoport, S. M. (1976) *Biochem. J. 154,* 449-469
Salter, M., Knowles, R. G. & Pogson, C. I. (1986) *Biochem. J. 234,* 635-647
Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology,* Addison-Wesley, Reading, Massachusetts
Small, J. R. & Fell, D. A. (1989) *J. Theor. Biol. 136,* 181-197
Sorribas, A. & Bartrons, R. (1986) *Eur. J. Biochem. 158,* 107-115
Stuart, F., Porteous, D. J., Flint, H. J. & Kacser, H (1986) *J. Gen. Microbiol. 132,* 1159-1166
Tager, J. M., Groen, A. K., Wanders, R. J. A., Duszynski, J., Westerhoff, H. V. & Vervoorn, R. C. (1983) *Biochem. Soc. Trans. 11,* 40-43
Torres, N., Mateo, F., Meléndez-Hevia, E. & Kacser, H. (1986) *Biochem. J. 234,* 169-174
Torres, N., Mateo, F. & Meléndez-Hevia, E. (1988*a*) *FEBS Letters 233,* 83-86
Torres, N., Mateo, F., Sicilia, J. & Meléndez-Hevia (1988*b*) *Int. J. Biochem. 20,* 161-165
Walsh, K. & Koshland, D. E., Jr. (1984) *J. Biol. Chem. 259,* 9649-9654
Wanders, R. J. A., Meijer, A. J., van Roermund, C. M., Groen, A. K., Lof, V. & Tager, J. M. (1983) *Biochem. Soc. Trans. 11,* 89-90
Wanders, R. J. A., van Roermund, C. W. T. & Meijer, A. J. (1984*a*) *Eur. J. Biochem. 142,* 247-254
Wanders, R. J. A., Groen, A. K., van Roermund, C. W. T. & Tager, J. M. (1984*b*) *Eur. J. Biochem. 142,* 417-424
Welch, G. R. (1977) *Prog. Biophys. Molec. Biol. 32,* 103-191
Werner, A. & Heinrich, R. (1985) *Biomed. Biochim. Acta 44,* 185-212

Part III

INTERCONVERTIBLE ENZYMES IN METABOLIC CONTROL

Chapter 12

Zero-order Ultrasensitivity in Interconvertible Enzyme Systems

ALBERT GOLDBETER and DANIEL E. KOSHLAND, JR.

HOW TO GENERATE thresholds is an important question in cellular metabolism as well as in other fields of biology. Thresholds are essential if large changes in response are to be elicited by relatively small changes in stimulus, i.e. substrate or effector in the case of enzymes (Koshland *et al.*, 1982). An important role is nevertheless retained in metabolic systems by other reactions in the pathway since these will determine whether any abrupt change is propagated beyond the threshold-generating step. Control of flux indeed appears to be distributed over the various steps of a pathway (Kacser & Burns, 1973; Westerhoff *et al.*, 1984). The reversible shutting down of the flux above a suprathreshold stimulation should, however, prove a most efficient way of generating sharp transitions under steady-state conditions.

The most common thresholds in biochemical systems are produced by allosteric enzymes whose dose-response curves are characterized by Hill coefficients larger than unity (Monod *et al.*, 1965; Koshland *et al.*, 1966). These curves, however, are moderately steep, as most Hill coefficients rarely exceed the value of 2 or 3. Sharper thresholds occur in all-or-none transitions associated with bistability, when a system abruptly switches between two stable steady states which coexist under the same conditions. Such a situation has been observed in several biochemical reactions (Degn, 1968; Naparstek *et al.*, 1973; Eschrich *et al.*, 1973) as a result of substrate inhibition or activation of an enzyme by a reaction product in the presence of a constant amount of substrate (the latter type of regulation can also give rise to oscillatory behaviour once the substrate level is allowed to vary).

Sharp threshold phenomena may also originate from covalent modification. This result could be of wide significance, given the ubiquitous role of covalent modification in normal and pathological cellular processes (Krebs & Beavo, 1979; Nestler & Greengard, 1984; Cohen, 1983; Hunter, 1987). The purpose of this chapter is to concisely review the various aspects of steep transitions that can occur in the dynamics of interconvertible enzyme systems.

Albert Goldbeter ● Faculté des Sciences, Université Libre de Bruxelles, Campus Plaine, C.P. 231, B-1050 Brussels, Belgium ❖ Daniel E. Koshland, Jr. ● Department of Biochemistry, University of California, Berkeley, California 94720, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

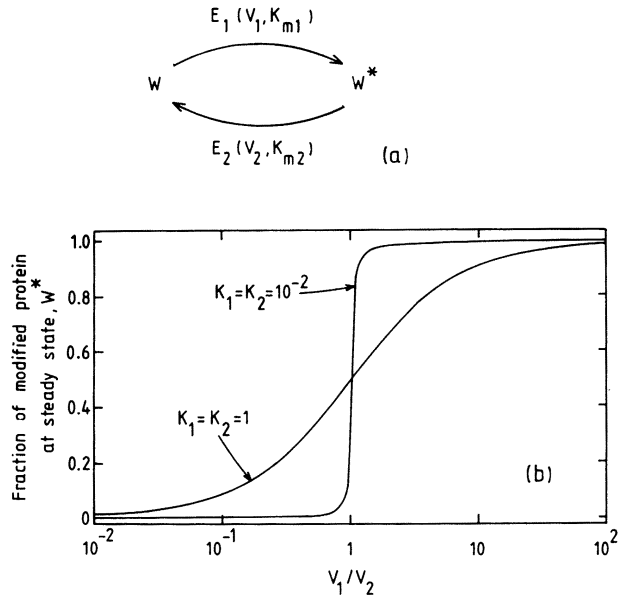


Figure 1. Monocyclic system of covalent modification (*a*) and transition of the target protein W into its modified form W* as a function of the ratio of maximum rates V_1/V_2 of the converter enzymes E_1 and E_2 (*b*). The curves are established for two values of the reduced Michaelis constants $K_1 = K_{m1}/W_T$, $K_2 = K_{m2}/W_T$, where $W_T = W+W^*$ (redrawn from Goldbeter & Koshland, 1981).

Zero-order ultrasensitivity in covalent modification

A conspicuous difference between regulation of enzyme activity by allosteric transitions and by covalent modification is that the latter requires the continuous expenditure of energy. Interconvertible enzyme systems are further characterized by the fact that one enzyme (a kinase, in the case of phosphorylation) catalyses the covalent modification of a protein substrate while another enzyme (a phosphatase, in the case of dephosphorylation) catalyses the reverse process; the two catalytic activities are generally, but not necessarily, carried by distinct enzymes. Therefore, in the presence of non limiting amounts of appropriate cofactors, interconvertible enzyme systems evolve to a nonequilibrium steady state.

One wishes to determine how the amount of protein modified at steady state will vary when the ratio of modification versus demodification rates increases. To facilitate the following discussion, we shall focus on the particular, most common case, i.e. protein phosphorylation. All results presented below hold, however, for other cases of covalent modification such as methylation, ADP-ribosylation, etc. for which the converter enzymes differ from the kinase and phosphatase associated with reversible phosphorylation.

The basic interconvertible enzyme system is represented in Fig. 1*a*. It consists in a protein W that is phosphorylated into W* by a protein kinase (enzyme E_1) and dephosphorylated by a phosphatase (E_2). The kinetic analysis of interconvertible enzyme systems has been pioneered by Stadtman & Chock (1977, 1978; Chock & Stadtman, 1977). An alternative approach followed by Goldbeter & Koshland (1981) rests on the expression of the amount of protein modified at steady state as a function of the ratio V_1/V_2 of phosphorylation versus dephosphorylation rates (see Fig. 1*a*), where V_1 and V_2 denote the maximum values of the modification and demodification rates that can be reached in the presence of a given amount of effector or in its absence.

Rather than incorporating into the expression of V_1/V_2 the regulatory effect exerted by an activator of E_1 and/or an inhibitor of E_2 so that W* is given from the outset as a complicated function of the effector concentration, our approach consists in two successive stages. First, the variation of W^* is expressed as a function of V_1/V_2; in a second stage, the dependence of V_1/V_2 on the level of some effector J controlling the converter enzymes is determined. Combining the two informations eventually yields the dependence of W* as a function of effector J. This two-step method has the advantage of clarifying the respective contributions made to the overall sensitivity by the covalent modification reactions and by the control exerted by the effector on the two converter enzymes.

When the complexes formed by the kinase and phosphatase with the target protein can be neglected, e.g. at large values of the target protein concentration W_T, the steady-state fraction of modified protein, denoted W^*, is given as a function of the ratio V_1/V_2 by the solution of a second-degree equation in the form of eqn. (1) (see Goldbeter & Koshland, 1981):

$$W^* = \frac{\phi + \left[\phi^2 + 4K_2\left(\frac{V_1}{V_2} - 1\right)\left(\frac{V_1}{V_2}\right)\right]^{1/2}}{2\left(\frac{V_1}{V_2} - 1\right)} \tag{1}$$

with

$$\phi = \left(\frac{V_1}{V_2} - 1\right) - K_2\left(\frac{K_1}{K_2} + \frac{V_1}{V_2}\right)$$

where K_1 and K_2 are the Michaelis constants K_{m1}, K_{m2} of the modifying enzymes E_1 and E_2, divided by the total amount of target protein W_T (see Fig. 1*a*). At this stage, we do not yet specify how the ratio V_1/V_2 is controlled by the effector (see section 4 below).

A striking result of this analysis is that the steady-state fraction of modified protein varies in a very steep manner with the ratio V_1/V_2 when K_1 and/or K_2 are much lower than unity, i.e. in conditions where the kinase and/or the phosphatase become saturated by their substrate (Fig. 1*b*). Accordingly, eqn. (1) indicates that in the limit of vanishingly small values of $K_1 = K_2$, W^* tends to zero when $V_1 < V_2$, and towards unity when $V_1 > V_2$.

The advantage of expressing W* in terms of V_1/V_2 becomes apparent in conditions where the converter enzymes are far from saturation by the substrate. Then, K_1 and K_2 are much larger than unity and W^* is given by eqn. (2) (Goldbeter & Koshland, 1981):

$$W^* = \frac{\frac{V_1}{V_2}}{\frac{K_1}{K_2} + \frac{V_1}{V_2}} \tag{2}$$

This expression shows that the curve of W^* versus V_1/V_2 will acquire the form of the traditional hyperbolic curve obtained for the saturation or velocity of Michaelis-Menten enzymes as a function of the substrate concentration (in semi-logarithmic plot, as in Fig. 1*b*, such curve has the appearance of a shallow sigmoid).

Therefore, V_1/V_2 is the natural parameter to use for comparing covalent modification kinetics with that of Michaelian or allosteric enzymes. When expressing W^* as a function of V_1/V_2, the modification curve varies from hyperbolic to sharply sigmoidal as the values of K_{m1} and K_{m2} are progressively reduced with respect to W_T, i.e. as the converter enzymes pass from the domain of first-order into that of zero-order kinetics (Fig. 1*b*). Hence the name of *zero-order ultrasensitivity* given to this phenomenon (Goldbeter & Koshland, 1981), any response sharper than Michaelian being referred to as *ultrasensitive*. It should be emphasized that the modification curve becomes steeper than Michaelian even before the modifying enzymes are saturated by their substrate (see Fig. 2). The steepness becomes more pronounced as the converter enzymes move deeper into the zero-order kinetic domain.

Clearly, thus, interconvertible systems are capable of generating threshold phenomena in physiological responses, in view of the fact that covalent modification is often associated with the activation or inactivation of a specific enzyme (Krebs & Beavo, 1979; Nestler & Greengard, 1984; Cohen, 1983; Hunter, 1987) or DNA-binding protein (Magasanik, 1988). A similar mode of regulation extends to receptors and ion channels (Lefkowitz & Caron, 1986; Levitan, 1985).

To compare the steepness of the modification transition with that of velocity curves generated by allosteric enzymes, it is useful to define a measure of the steepness of response curves as a function of a given stimulus. Such a measure, for allosteric enzymes, is given by the *cooperativity index* $R_S = S_{0.9}/S_{0.1}$ which is equal to the ratio of substrate (effector) concentrations yielding, respectively, 90% and 10% of the maximal saturation or velocity (Taketa & Pogell, 1965). The link between R_S and the Hill coefficient n_H is given by eqn. (3):

$$R_S = 81^{1/n_H} \tag{3}$$

The smaller R_S the steeper the response curve (for example, $R_S = 9$ and 3 for $n_H = 2$ and 4 respectively); R_S approaches unity — the transition tends to become vertical — when n_H goes to infinity.

A similar measure for interconvertible enzyme systems is provided by the coefficient R_V defined by eqn. (4) as the ratio of the value of V_1/V_2 yielding 90% of protein modified at steady state, divided by the value of V_1/V_2 yielding $W^*=0.1$ (Goldbeter & Koshland, 1981):

$$R_V = \frac{(V_1/V_2)_{0.9}}{(V_1/V_2)_{0.1}} \tag{4}$$

An analytical expression for R_V can readily be obtained from the expression that yields the value of V_1/V_2 corresponding to a particular value of W^* at steady state (Goldbeter & Koshland, 1981).

The relative steepness of response curves generated by allosteric enzymes and by enzymes subjected to covalent modification is shown in Fig. 2. The cooperativity index R_S is given as a function of the Hill coefficient characterizing positive cooperativity of the

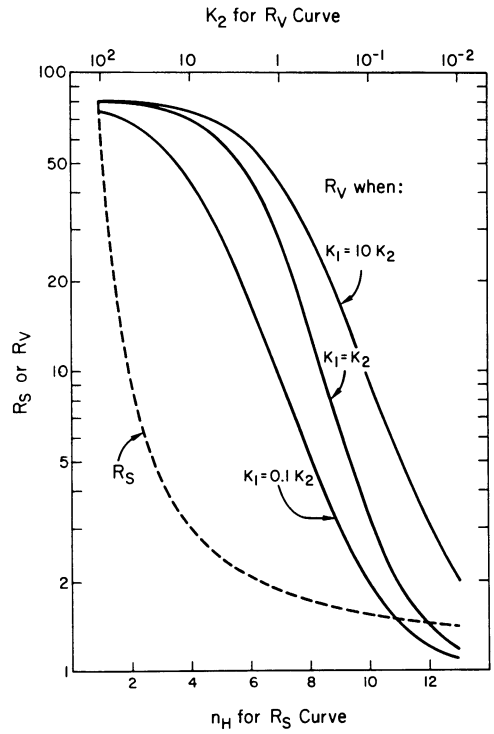


Figure 2. Comparison of sensitivity in covalent modification (R_V) and allosteric regulation (R_S); the sensitivity indices are defined in the text (redrawn from Goldbeter & Koshland, 1981).

allosteric enzyme, while the corresponding index R_V for the interconvertible enzyme system is given as a function of the reduced Michaelis constants of the modifying enzymes, K_1 and K_2 (the effect of unequal Michaelis constants is also shown).

The figure indicates that for $K_1 = K_2 = 1$ already, the steepness of the transition curve for covalent modification approaches that of an allosteric enzyme whose Hill coefficient equals 2. For $K_1 = K_2 = 10^{-2}$, the steepness in covalent modification corresponds to Hill coefficients larger than 12. Steeper curves corresponding to even larger Hill coefficients can be generated with lower values of K_1 and K_2. Conditions favouring maximum steepness have also been investigated by Cárdenas & Cornish-Bowden (1989; see also Chapter 14 of this book) in a recent analysis of the monocyclic system of covalent modification.

In the above treatment, W^* was obtained as a function of V_1/V_2 by solving a second-degree equation. The latter transforms into a third-degree equation when the concentration of enzyme-substrate complexes ceases to be negligible with respect to that of the target protein, or in the presence of significant inhibition of converter enzymes by their products (Goldbeter & Koshland, 1981). On the other hand, the equation for W^* is of the fourth degree when the kinase also acts on a second target protein which competes for the same free enzyme; zero-order ultrasensitivity also occurs in these conditions but the threshold value of (V_1/V_2) for the substrate W is modified by the presence of the second substrate.

Have the theoretical predictions on zero-order ultrasensitivity been confirmed by experimental observations? So far, only three enzyme systems have been investigated. These are

isocitrate dehydrogenase in *E. coli* (LaPorte & Koshland, 1983), a synthetic nonapeptide (Shacter *et al.*, 1984*a*), and glycogen phosphorylase (Meinke *et al.*, 1986; see also Chapter 15 by Edstrom and others in this book). In all instances, a steady-state modification curve steeper than Michaelian has been obtained, but the increase in steepness resulting from an increased amount of target protein has only been demonstrated in the first and last of these interconvertible systems, by comparison of covalent modification at different substrate concentrations.

One reason why experimental support has not been obtained on a larger scale has to do with the type of experiment that needs to be performed *in vitro*. While *in vivo* the two converter enzymes are generally present simultaneously, *in vitro*, few experiments on covalent modification are performed in the presence of the two converter enzymes. Thus, the kinetics of phosphorylation is usually determined in the presence of the kinase, while dephosphorylation is studied in the sole presence of phosphatase. Although these steps are essential for a thorough characterization of the kinetics of each converter enzyme, it is important to realize that threshold phenomena will only arise in the simultaneous presence of both enzymes, even though the kinase and the phosphatase might individually possess Michaelian behaviour. Therefore, to demonstrate steep transitions, experiments must necessarily be of the sort exemplified by the studies on ultrasensitivity in covalent modification mentioned above. These were performed with varying amounts of target protein in the presence of the two converter enzymes.

Propagation of Ultrasensitivity in Enzyme Cascades

A peculiar feature of biochemical systems regulated by covalent modification is that they often are organized in cascades: the protein modified in one cycle catalyses the modification of another protein in a second cycle, and this second modified protein may in turn be involved as converter enzyme in yet another cycle of covalent modification... This type of multicyclic cascade organization is well exemplified by glycogen metabolism (Cohen, 1983) and by the regulation of glutamine synthetase (Rhee *et al.*, 1989; see also Chapter 13 by Chock, Rhee and Stadtman, and others in this book). The latter system provides a beautiful example, since interconvertible cascades are implicated in nitrogen metabolism both at the levels of enzyme and genetic regulation (Magasanik, 1988; Rhee *et al.*, 1989).

The question arises as to whether the amplification gained through zero-order ultrasensitivity in one cycle of a cascade can be propagated and further enhanced in subsequent cycles. A detailed analysis (Goldbeter & Koshland, 1982) shows that such a phenomenon is indeed possible: in the bicyclic cascade of Fig. 3*a*, an effector J activates enzyme E_1 of the first cycle; as a result, W is transformed into W* as J increases. Given that W* catalyses the transformation of Z into Z*, Z^* will also rise with J. It can be seen from Fig. 3*b* that the transition curve for Z^* can be steeper than that obtained for W^*. Moreover, for the parameter values considered, the rise in Z^* precedes the increase in W^*. Therefore, in these conditions, the transition from Z to Z* takes place with increased sensitivity on several counts: it occurs at lower values of the effector, over a smaller range of J values, and the rise in Z^* may be faster than that in W^*. The acceleration and the occurrence of the

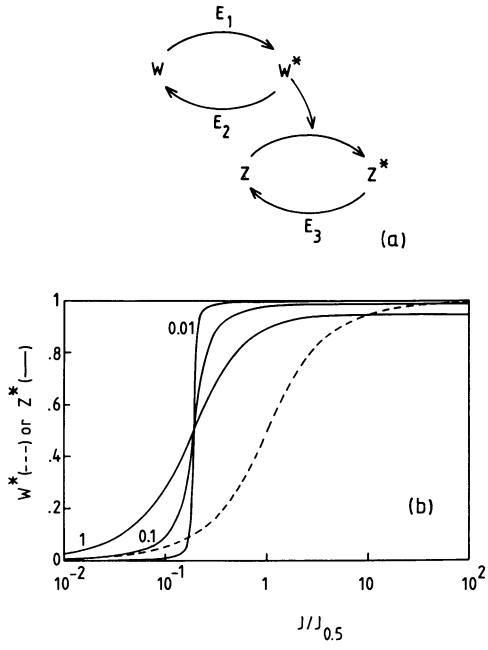


Figure 3. Bicyclic cascade of covalent modification (*a*) where the product of the first cycle, W*, catalyses the modification of the second target protein Z into Z*. The modification transition is shown in (*b*) for the two target proteins, as a function of an effector J that activates enzyme E_1; the curves are established for three values of the reduced Michaelis constants of the second cycle (redrawn from Goldbeter & Koshland, 1982).

transition at lower and lower effector levels were emphasized by Stadtman & Chock (1977, 1978; Chock & Stadtman, 1977) in their analysis of multicyclic cascades; sensitivity amplification due to the zero-order effect was not obtained in that analysis, given that it was restricted to the situation in which converter enzymes possess first-order kinetics.

The situation illustrated in Fig. 3*b* is, however, not generic. The enhancement of sensitivity in interconvertible enzyme cascades depends on the kinetic parameters that characterize converter enzymes in each cycle of the cascade. Thus, the sensitivity gained in one cycle will be reduced or even lost, and thereby transformed into subsensitivity, by subsequent cycles if the kinetic parameters of the latter are outside the range producing zero-order ultrasensitivity (Goldbeter & Koshland, 1982).

Interplay between Multi-step and Zero-order Ultrasensitivity

Until now, not much has been said about the manner in which a given effector controls the ratio of modification rates V_1/V_2 which determines the value of W* attained at steady state. In the simplest case illustrated by the action of cyclic AMP on a protein kinase, the effector activates enzyme E_1 and thereby increases V_1 (mechanism I). Alternatively, the ratio V_1/V_2 may rise due to the (competitive or non-competitive) inhibition of E_2 by J (mechanism II). A third possibility is that of dual control in which J activates E_1 and inhibits E_2 (mechanism III). An important consequence of the latter type of control is that even if the two regulations proceed in a non-cooperative manner, their concomitance can by itself result

in increased sensitivity. To underline the fact that such amplification originates from the multiple controls exerted by a given effector, this phenomenon was termed *multi-step ultrasensitivity* (Koshland *et al.*, 1982; Goldbeter & Koshland, 1984).

An effector activating a kinase and inhibiting a phosphatase in a Michaelian manner can already produce maximal ultrasensitivity equivalent to that of an allosteric enzyme characterized by a Hill coefficient of 2. Multi-step ultrasensitivity is favoured when the phosphatase is inhibited before the kinase becomes activated as the effector increases (Goldbeter & Koshland, 1984). A similar condition for optimal amplification was also noted by Cárdenas & Cornish-Bowden (1989).

Dual control of converter enzymes in one or more cycles of an interconvertible enzyme cascade can further increase the amplification provided by zero-order ultrasensitivity. The phenomenon can also give rise to staircase transitions when the effector level progressively increases (Goldbeter & Koshland, 1984). This occurs, for example, in the bicyclic cascade of Fig. 3*a* when the thresholds for the modification transitions of the successive target proteins are well separated as a function of the effector level, with W* being turned on before Z*.

Energy Expenditure at Steady State in Interconvertible Enzyme Systems

Characteristic of regulation by covalent modification is the continuous expenditure of energy required to maintain a particular fraction of modified protein at steady state. How does this energy expenditure vary with the value of W^*? A comparative analysis indicates (Goldbeter & Koshland, 1987) that the variation of ATP consumption markedly depends on the mode of control of the converter enzymes by the effector J whose increase brings about the rise in W^*.

Fig. 4 shows how the energy expenditure at steady state changes with W^* when the converter enzymes are controlled according to one of the three mechanisms I-III discussed in the preceding section. The curve takes the form of an increasing or decreasing hyperbola, depending on whether the rise in V_1/V_2 is brought about by activation of the kinase or inhibition of the phosphatase; the two curves become linear when the unregulated enzyme of the pair (the phosphatase in mechanism I and the kinase in mechanism II) functions in the first-order kinetic domain. Finally, a bell-shaped curve is obtained for energy expenditure as

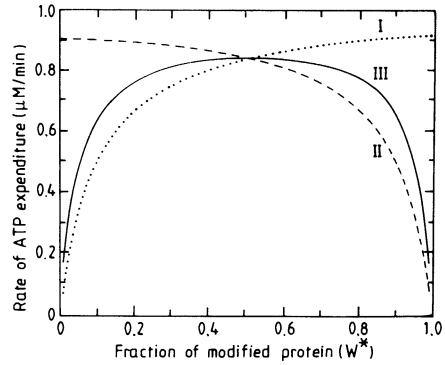


Figure 4. Energy expenditure at steady state as a function of the fraction of modified protein in the monocyclic system of Fig. 1*a*, when the rise in the ratio V_1/V_2 is brought about by an increase in V_1 (I), a decrease in V_2 (II), or both effects (III) (redrawn from Goldbeter & Koshland, 1987).

a function of W^* when the converter enzymes are controlled according to the dual mechanism III in which the effector activates the kinase and inhibits the phosphatase (Goldbeter & Koshland, 1987).

These results account for the finding of Shacter *et al.* (1984*b*) that energy expenditure increases linearly with the fraction of nonapeptide phosphorylated at steady state: in this system, indeed, the increase in phosphorylation is achieved through activation of the kinase by cyclic AMP (mechanism I), while the phosphatase operates with first-order kinetics. The theoretical analysis (Fig. 4; also Goldbeter & Koshland, 1987) indicates, however, that the situation of a linear increase in ATP consumption should not hold for all converter enzyme systems.

Evaluation of the ATP consumed in the many reactions controlled by covalent modification shows that the cumulated energy expended in this manner is far from being negligible with respect to the total ATP flux (Goldbeter & Koshland, 1987). Curve III in Fig. 4 indicates that dual control of the two converter enzymes by the same effector provides a particularly effective mode of control which allows both for increased sensitivity and reduced energy expenditure at steady state, when the fraction of target protein reversibly varies from a low to a high value in response to changed conditions.

Concluding Remarks

Together, the above results indicate that covalent modification provides a most sensitive mode of metabolic control. Thresholds associated with zero-order ultrasensitivity indeed allow the amplification of small changes in some effector concentration. This sensitivity amplification can be propagated in interconvertible enzyme cascades, and further enhanced when the effector regulates the two converter enzymes in a monocyclic system of covalent modification, or several converter enzymes in more than one cycle of a cascade. Furthermore, cascades allows for rate and magnitude amplification, since under suitable conditions successive cycles of covalent modification will be turned on more and more rapidly, at lower and lower values of the effector concentration. The analysis of interconvertible enzyme systems permits to determine both the conditions that must be satisfied to optimize the sensitivity of this mode of control, and the amount of energy that must be expended to maintain a given level of protein modified at steady state.

Acknowledgments: This work was supported by the Belgian National Incentive Programme for Fundamental Research in the Life Sciences (Convention BIO/08) launched by the Science Programming Services of the Prime Minister's Office (SPPS), and by a grant from the National Fund for Scientific Research (FNRS) in the framework of a FNRS-NSF program.

REFERENCES

Cárdenas, M. L. & Cornish-Bowden, A. (1989) *Biochem. J. 257,* 339-345
Chock, P. B. & Stadtman, E. R. (1977) *Proc. Natl. Acad. Sci. USA 74,* 2766-2770
Cohen, P. (1983) *Phil. Trans. Roy. Soc. Lond. B 302,* 13-25
Degn, H. (1968) *Nature 217,* 1047-1050

Eschrich, K., Schellenberger, W. & Hofmann, E. (1980) *Arch. Biochem. Biophys. 205,* 114-121
Goldbeter, A. & Koshland, D. E., Jr. (1981) *Proc. Natl. Acad. Sci. USA 78,* 6840-6844
Goldbeter, A. & Koshland, D. E., Jr. (1982) *Quart. Rev. Biophys. 15,* 555-591
Goldbeter, A. & Koshland, D. E., Jr. (1984) *J. Biol. Chem. 259,* 14441-14447
Goldbeter, A. & Koshland, D. E., Jr. (1987) *J. Biol. Chem. 262,* 4460-4471
Hunter, T. (1987) *Cell 50,* 823-829
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exper. Biol. 27,* 65-104
Koshland, D. E., Jr., Némethy, G. & Filmer, D. (1966) *Biochemistry 5,* 365-385
Koshland, D. E., Jr., Goldbeter, A. & Stock, J. B. (1982) *Science 217,* 220-225
Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48,* 923-959
LaPorte, D. C. & Koshland, D. E., Jr. (1983). *Nature 305,* 386-290
Lefkowitz, R. J. & Caron, M. G. (1986) *J. Mol. Cell. Cardiol. 18,* 885-895
Levitan, I. B. (1985) *J. Membr. Biol. 87,* 177-190
Magasanik, B. (1988) *Trends Biochem. Sci. 13,* 475-479
Meinke, M. H., Bishop, J. S. & Edstrom, R. D. (1986) *Proc. Natl. Acad. Sci. USA 83,* 2865-2868
Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol. 12,* 88-118
Naparstek, A., Romette, J. L., Kernevez, J. P. & Thomas, D. (1973) *Nature 249,* 490-491
Nestler, E. J. & Greengard, P. (1984) *Protein Phosphorylation in the Nervous System,* Wiley, New York
Rhee, S. G., Chock, P. B. & Stadtman, E. R. (1989) *Adv. Enzymol. 62,* 37-92
Shacter, E., Chock, P. B. & Stadtman, E. R. (1984*a*) *J. Biol. Chem. 259,* 12252-12259
Shacter, E., Chock, P. B. & Stadtman, E. R. (1984*b*) *J. Biol. Chem. 259,* 12260-12264
Stadtman, E. R. & Chock, P. B. (1977) *Proc. Natl. Acad. Sci. USA 74,* 2761-2765
Stadtman, E. R. & Chock, P. B. (1978) *Curr. Topics Cell. Reg. 13,* 53-95
Taketa, K. & Pogell, B. M. (1965) *J. Biol. Chem. 240,* 651-652
Westerhoff, H. V., Groen, A. K. & Wanders, R. J. A. (1984) *Biosci. Rep. 4,* 1-22

Chapter 13

Metabolic Control by the Cyclic Cascade Mechanism: a Study of *E. coli* Glutamine Synthetase

P. BOON CHOCK, SUE GOO RHEE and EARL R. STADTMAN

THE CYCLIC CASCADE model was developed through detailed studies on the regulation of *Escherichia coli* glutamine synthetase. This enzyme catalyses the biosynthesis of glutamine and since the amide nitrogen atom of glutamine is a preferred nitrogen source for the biosynthesis of virtually all amino acids, purine and pyrimidine nucleotides, NAD and glucosamine-6-phosphate, it is rigorously regulated in bacteria which synthesize their own amino acids. [For reviews, see Stadtman & Ginsburg (1974), Stadtman & Chock (1978), Chock *et al.*, (1980), Rhee *et al.*, (1985).]

Cyclic Cascade Regulation of Glutamine Synthetase Activity

Extensive investigation on the regulation of the *E. coli* glutamine synthetase revealed that in addition to cumulative feedback control, the activity of the dodecameric enzyme is mainly regulated by covalent interconversion of the enzyme. The latter involved a bicyclic cascade which is comprised of two protein nucleotidylation cycles. On one cycle, glutamine synthetase is reversibly adenylylated at Tyr-397 on each subunit. On the other cycle, a regulatory protein P_{II} is uridylylated and deuridylylated at a specific tyrosine residue of each P_{II} subunit (see the upper two cycles of Fig. 1).

The adenylylation and deadenylylation of glutamine synthetase are catalysed at separate sites (designated in Fig. 1 as AT_a and AT_d, respectively) on a single adenylyltransferase with molecular mass 110 kDa. The adenylylation reaction involves the covalent attachment of the adenylyl moiety from ATP to the hydroxyl group of Tyr-397 via a phosphodiester linkage. Since glutamine synthetase contains twelve identical subunits and the adenylylated subunits are catalytically inactive under most physiological conditions, the specific activity of

P. Boon Chock, Sue Goo Rhee and Earl R. Stadtman ● Laboratory of Biochemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

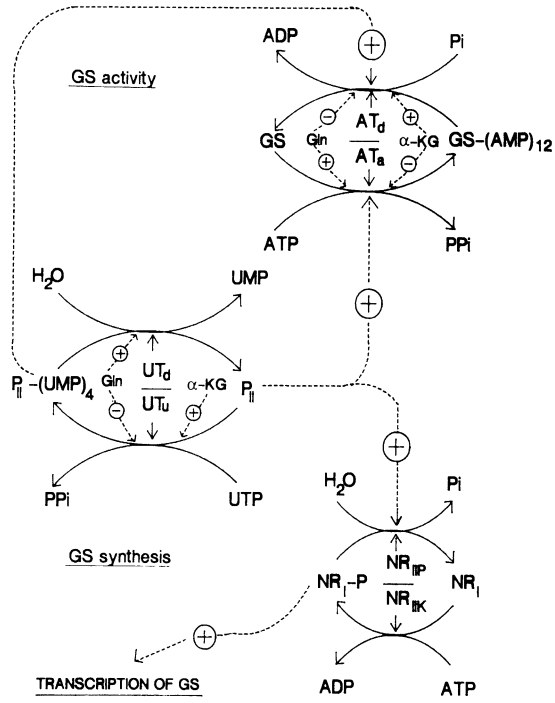


Figure 1. The cyclic cascade of glutamine synthetase regulation. Interrelationship between the uridylylation cycle, the adenylylation cycle, and the phosphorylation cycle; the reciprocal controls of these interconversions by L-glutamine (Gln) and α-ketoglutarate (α-KG) are shown; ⊕ indicates stimulation, ⊖ indicates inhibition. Abbreviations: GS, glutamine synthetase; P_{II}, regulatory protein, AT_a and AT_d, adenylyltransferase activity that catalyses the adenylylation and deadenylylation reaction, respectively; UT_d, uridylyl-removing enzyme or deuridylylation enzyme; UT_u, uridylyltransferase; NR_I, *glnG* product (also known as NTRC); NR_{IIK} and NR_{IIP}, *glnL* product (also known as NTRB) catalysing phosphorylation and dephosphorylation respectively of NR_I.

glutamine synthetase is almost inversely proportional to the average number $\bar{n}$ of adenylylated subunits per dodecamer (Stadtman *et al.*, 1968). Although the adenylylation process is reversible (Mantel & Holzer, 1970), under physiological conditions, the adenylyl group is removed from glutamine synthetase by site AT_d catalysed phosphorolysis of the adenylyl-O-tyrosyl bond to yield ADP and the deadenylylated glutamine synthetase (Anderson & Stadtman, 1970). Since the sites AT_a and AT_d are both located in a single polypeptide, without proper control these two reactions would be intimately coupled and glutamine synthetase would undergo senseless cycling between its adenylylated and deadenylylated forms. The net result of this cycling is simply phosphorolysis of ATP to form ADP and inorganic pyrophosphate. Such futile cycling is prevented by the linkage of the adenylylation cycle with another nucleotidylation cycle in which a regulatory protein, P_{II}, for the adenylylation/deadenylylation reaction undergoes interconversion between its uridylylated and deuridylylated forms.

The uridylylation and deuridylylation of P_{II} are catalysed at separate catalytic sites (designated in Fig. 1 as UT_u and UT_d, respectively) on another bifunctional enzyme, uridylyl transferase, with a molecular mass of 95 kDa (Garcia & Rhee, 1983; Brown *et al.*, 1971). The uridylylation of P_{II} involves the phosphodiester linkage of the uridylyl moiety from UTP to the hydroxyl group of a specific tyrosyl residue in each of the four identical subunits of molecular mass 11 kDa. The deuridylylation is the hydrolytic cleavage of the uridylyl-O-tyrosyl bond to form UMP and the unmodified P_{II}. Since a single polypeptide catalyses both uridylylation and deuridylylation of P_{II}, strict regulation is needed. Otherwise, the two

reactions would couple and result in cyclic interconversion of P_{II} between its uridylylated and deuridylylated forms, with concomitant hydrolysis of UTP to UMP and inorganic pyrophosphate. This potential futile cycle is prevented by the regulation of P_{II} interconversion by metabolites.

The uridylylation cycle and the adenylylation cycle are coupled due to the facts that the unmodified form of P_{II} stimulates the activity of AT_a for adenylylating glutamine synthetase, whereas the uridylylated form of P_{II} is required to activate the site AT_d activity (Brown *et al.*, 1971). This cascade functions under normal physiological conditions as a dynamic processing unit in which the interconvertible proteins undergo continual modification and demodification. For any given metabolic condition, a steady state of fractional modification of the interconvertible enzymes will be established. Thus, the fractional modification of P_{II} and of glutamine synthetase are determined by the concentrations of various metabolites that can influence the activities of the converter enzymes, namely, sites AT_a, AT_d, UT_u, and UT_d (Stadtman *et al.*, 1979). In fact, about 40 metabolites have been shown to affect one or more of these enzymes (Stadtman & Chock, 1978). Among them, α-ketoglutarate and glutamine play a dominant role in the regulation of glutamine synthetase (Stadtman & Ginsburg, 1974; Stadtman & Chock, 1978; Rhee *et al.*, 1978). As depicted in Fig. 1, glutamine stimulates the adenylylation of glutamine synthetase and the deuridylylation of $P_{II}(UMP)_n$, whereas it inhibits the deadenylylation of glutamine synthetase-$(AMP)_n$ and the uridylylation of P_{II}. Conversely, α-ketoglutarate inhibits the adenylylation of glutamine synthetase, but it stimulates the deadenylylation of glutamine synthetase-$(AMP)_n$ and the uridylylation of P_{II}. In other words, the activity of site AT_a which leads to the inactivation of glutamine synthetase is stimulated by glutamine and inhibited by α-ketoglutarate, whereas the activities of site AT_d and site UT_u which lead to the reactivation of glutamine synthetase are stimulated by α-ketoglutarate and inhibited by glutamine. The site UT_d activity which initiates the inactivation cascade of glutamine synthetase is stimulated by glutamine but not inhibited by α-ketoglutarate. The beauty of these reciprocal effects of glutamine and α-ketoglutarate can be fully appreciated if one considers that α-ketoglutarate is a precursor of glutamine and therefore the ratio of glutamine to α-ketoglutarate will vary in response to variations in the levels of ammonia. Consequently, the specific activity of glutamine synthetase will vary rapidly in response to the availability of ammonia as shown in *in vitro* and *in vivo* experiments (Senior, 1975; Rhee *et al.*, 1978; Mura *et al.*, 1981).

The mechanism by which the bifunctional converter enzyme, adenylyltransferase, catalyses the adenylylation and deadenylylation in the presence of allosteric effectors glutamine and α-ketoglutarate has been deduced from steady-state kinetic data (Rhee *et al.*, 1988, 1989). The effects of glutamine on the adenylylation reaction are to enhance the affinities of ATP and P_{II} by about 20- and 10-fold, respectively, and to increase the catalytic rate constant by about 20-fold. P_{II} increases the affinities of glutamine synthetase and glutamine by 13- and 10-fold, respectively. In the deadenylylation reaction, $P_{II}(UMP)_n$ is required and it also enhances the affinities of α-ketoglutarate and ATP, an activator, by about 20- and 8-fold respectively. ATP and α-ketoglutarate enhance each other's binding affinity by a factor of 140 and α-ketoglutarate increases the affinity of glutamine synthetase$(AMP)_n$ by about 22-fold. The inhibition of the adenylylation and deadenylylation reactions by glutamine and

α-ketoglutarate derives from the fact that these two effectors decrease each other's binding affinity by a factor of 0.02. In the case of uridylylation, UTP binds to uridylyltransferase prior to P_{II} and the allosteric effector ATP enhances the affinity of α-ketoglutarate by about 10-fold. The maximum velocity for the reaction is activated 21-fold by α-ketoglutarate. Glutamine enhances the affinity of P_{II}(UMP) by 4-fold while it functions as an uncompetitive inhibitor with respect to P_{II}. It is interesting to note that glutamine and α-ketoglutarate do not affect each other's binding to the uridylyltransferase and α-ketoglutarate does not inhibit the deuridylylation reaction.

The efficiency of a multicyclic cascade system can be further enhanced by a pyramidal relationship for the concentration of the enzymes participating in the cascade. Under these conditions, the enzyme concentrations in the first cycle are lower than those in the next cycle, and the concentration of the converter enzyme is lower than that of the interconvertible enzyme it catalyses. It is worth noting that this pyramidal relationship exists in the bicyclic cascade of glutamine synthetase. The relative concentrations of glutamine synthetase (subunit), P_{II}, adenylyltransferase and uridylyltransferase were found to exist in a ratio of 411 : 42 : 2.6 : 1.0 for *E. coli* K12 grown under derepressed growth conditions (Rhee *et al.*, 1989).

The Cyclic Cascade Model

It is evident that the analysis of biological cyclic cascades is extremely complex. However, the results of the in-depth investigation on the cyclic cascade that regulates the activity of glutamine synthetase allows one to formulate a simplified model which contains only the essential features of the interconvertible enzyme systems. Fig. 2 depicts a monocyclic cascade of the model discussed. It consists of a forward and a reverse cascade. In the forward cascade, an inactive converter enzyme, E_i, is activated by an allosteric effector e_1 to its active state, E_a. E_a then catalyses the conversion of an interconvertible enzyme, I, from its unmodified form, o-I, to its modified form, m-I. In the regeneration cascade, the inactive converter enzyme, R_i, is activated by an allosteric effector, e_2. This activated converter enzyme, R_a, then catalyses the conversion of the modified interconvertible enzyme to its original form, o-I. Dynamic coupling of these forward and reverse cascades leads to a steady-state in which there is cyclic interconversion between o-I and m-I, and the concomitant hydrolysis of ATP to ADP and inorganic phosphate. The monocyclic cascade is extended to multicyclic cascade when one form of the interconvertible enzyme function as a

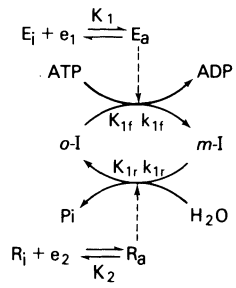


Figure 2. Schematic representation of a monocyclic cascade: K_1, K_2, K_{1f} and K_{1r}, are dissociation constants for e_1 from E_a, e_2 from R_a, o-I•E_a and m-I•R_a respectively; k_{1f} and k_{1r} are specific rate constants for the reaction designated.

converter enzyme for the next interconvertible enzyme and so on (Stadtman & Chock, 1977*b;* Chock & Stadtman, 1977).

In the theoretical analysis, it has been ascertained that the covalent modification of enzymes does not function simply as an *on-off* switch for various metabolic pathways, but rather that it is part of a dynamic process in which the fractional activities of the interconvertible enzymes can be varied progressively over a wide range (Stadtman & Chock, 1977*b;* 1978). This concept derived from the observation that the adenylylation of glutamine synthetase is not an all-or-none process; instead, a steady-state is established and its level is modulated by the concentration of effectors involved (Segal *et al.,* 1974). Similar observations have been reported for the mammalian pyruvate dehydrogenase complex (Pettit *et al.,* 1975). Furthermore, the ATP concentration is maintained in excess relative to the enzymes involved and at a fairly constant level. Other assumptions used include (i) the formation of the enzyme-enzyme and enzyme-effector complexes proceed via a rapid equilibrium mechanism, (ii) the concentration of the enzyme-enzyme complex is negligible compared to the concentrations of the active and inactive enzymes; and (iii) the concentrations of the allosteric effectors are maintained at constant levels for any metabolic state. With these assumptions, relatively simple equations can be derived to quantitate the behaviour of cyclic cascades.

Quantitative analyses of cyclic cascades, revealed (Chock *et al.,* 1980; Stadtman & Chock, 1977*b,* 1978; Chock & Stadtman, 1977) the following features:

1. They are endowed with an enormous capacity for signal amplification. As a consequence, they can respond effectively to concentrations of primary effector (such as e_1 in Fig. 2) well below the dissociation constant of the effector-enzyme complex;

2. They can modulate the amplitude of the maximal response that an interconvertible enzyme can accomplish even at saturating concentrations of allosteric effectors;

3. They can enhance the sensitivity of modification of the interconvertible enzyme to changes in the concentrations of allosteric effectors (i.e. they are capable of eliciting apparent positive and negative cooperativity in response to increasing concentrations of allosteric effectors);

4. They serve as biological integrators that can sense simultaneous fluctuations in the intracellular concentrations of numerous metabolites and adjust the specific activity of the interconvertible enzymes accordingly;

5. They are highly flexible with respect to allosteric regulation and are capable of exhibiting a variety of responses to primary allosteric stimuli;

6. They serve as rate amplifiers and therefore are capable of responding extremely rapidly to changes in metabolite levels (Chock & Stadtman, 1979; Stadtman & Chock, 1979).

Signal Amplification

This is a time-independent parameter defined (Stadtman & Chock, 1977*b;* 1978) as the ratio of the concentration of the primary allosteric effector required to attain a 50% activation of the converter enzyme to the concentration required to produce 50% modification of the interconvertible enzyme. This property derives from the fact that the signals (allosteric effectors) exert their effects on the target interconvertible enzymes through the catalytic actions of converter enzymes. Quantitatively, signal amplification is a multiplicative function of the kinetic parameters required to describe the cyclic cascade. As a result, small changes in several parameters can lead to enormous gains in signal amplification. Furthermore, because the number of parameters needed to describe the cascade increases with increasing numbers of cycles, the signal amplification potential of the last interconvertible enzyme in a multicyclic cascade increases exponentially with the number of cycles in the cascade. Thus, under certain conditions, it is feasible to attain an 800-fold signal amplification in the adenylylation/deadenylylation cascade of glutamine synthetase reconstituted in vitro (Rhee *et al.,* 1978). Moreover, experiments with permeabilized *E. coli* cells demonstrated that the bicyclic cascade of glutamine synthetase possesses higher signal amplification than the monocyclic cascade (Mura *et al.,* 1988). It should be pointed out that in unidirectional cascades, signal amplification is infinite because with sufficient time and ATP, all of the unmodified interconvertible enzyme will be converted to its modified form in response to any level of allosteric effector. Furthermore, it is noteworthy that the signal amplification described here is different from catalytic amplification, which is solely a function of the relative concentrations and catalytic efficiencies of the converter and interconvertible enzymes in the cascade. In many cascades, there exists a pyramidal increase in the concentrations of the cascade enzymes; that is, the concentration of the converter enzyme is significantly lower than that of its interconvertible enzyme substrate. Therefore, they also possess a high catalytic amplification potential.

Amplitude

Amplitude is defined (Stadtman & Chock, 1978) as the maximal value of fractional modification of the interconvertible enzyme attainable with saturating concentrations of an effector. By changing the magnitude of the cascade parameters, the amplitude can change smoothly from nearly 100% to almost 0%. Therefore, even at saturating levels of an allosteric effector, interconvertible enzymes do not function as *on-off* switches.

Sensitivity

Cyclic cascades can generate either apparent positive or negative cooperative responses of fractional modification (i.e. enzymic activity) of the interconvertible enzyme to increasing concentrations of an allosteric effector (Stadtman & Chock, 1987). These apparent coopera-

tivities can be attained from the synergistic or antagonistic effects that a single allosteric effector exerts on two or more steps in the cascade. Therefore, a sigmoidal response need not reflect positive cooperativity in the binding of an effector to multiple binding sites on the converter enzyme. Instead, it can be accomplished when an effector activates the forward converter enzyme and inactivates the reverse converter enzyme, or vice versa. Consequently, an effective way for obtaining high sensitivity is to have both forward and reverse converter enzyme activities combined in a single polypeptide such that binding of one effector can activate one activity while inactivating the other activity. Four such bifunctional enzymes involved in cyclic cascade systems have been isolated and characterized. They are the uridylyltransferase/uridylyl-removing enzyme activities that catalyse the uridylylation/deuridylylation of the P_{II} regulatory protein (Garcia & Rhee, 1983), the adenylyltransferase that catalyses the adenylylation/deadenylylation of glutamine synthetase (Caban & Ginsburg, 1976; Rhee *et al.*, 1978), a protein kinase/phosphatase that catalyses the phosphorylation and dephosphorylation of isocitrate dehydrogenase in *E. coli* (LaPorte & Koshland, 1982), and the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase that catalyses the synthesis and breakdown of fructose-2,6-bisphosphate (El-Maghrabi *et al.*, 1982; Van Schaftingen *et al.*, 1982). Conditions for maximizing a high sensitivity exerted by an effector to activate the forward and inhibit the reverse converter enzymic activities has also been reported (Cárdenas & Cornish-Bowden, 1989; see also Chapter 14 in this book). In addition, a sigmoidal response can be achieved by a monocyclic cascade in which the type II regulatory subunit of cAMP-dependent protein kinase inhibits the MgATP-dependent protein phosphatase (Jurgensen *et al.*, 1985). An apparent cooperative response can also occur when the converter enzymes are saturated by their interconvertible enzyme substrates (Goldbeter & Koshland, 1981; Shacter *et al.*, 1984).

Flexibility and Biological Integration

There are two aspects of flexibility that need to be considered when discussing the properties of cyclic cascades; namely, the flexibility for generating various allosteric control patterns and the flexibility in regulation by multiple metabolites. The scheme shown in Fig. 2 illustrates just one of many variations that can be derived by changing the nature of the interactions between the allosteric effectors, e_1 and e_2, and the converter enzymes, E and R (Stadtman & Chock, 1977*b*). Analysis of various mechanistic schemes shows that cyclic cascade can generate an array of regulatory patterns of fractional modification of the interconvertible enzyme in response to increasing effector concentration. These patterns differ with respect to their amplitude, signal amplification and sensitivity. In addition, the fact that a minimum of two converter enzymes and one interconvertible enzyme is needed to form a single interconvertible cycle, and each enzyme can be a separate target for one or more allosteric effectors, cyclic cascades provide a high degree of flexibility for metabolic input. Through allosteric interactions with the cascade enzymes, fluctuations in the concentration of numerous metabolites lead to automatic adjustments in the activities of the converter enzymes that determine the steady-state levels of fractional modification (specific

activity) of the interconvertible enzymes. In essence, cyclic cascades serve as biological integrators that can sense changes in the concentrations of innumerable metabolites and modulate the activities of pertinent enzymes accordingly.

Rate Amplification

Kinetic analysis of multicyclic cascades revealed that the rate of covalent modification of the last interconvertible enzyme in the cascade is a multiplicative function of the rate constants of all the reactions that lead to the formation of the modified enzyme (Stadtman & Chock, 1979). Therefore, following an initial lag period, cyclic cascades can function as rate amplifiers to generate an almost explosive increase in catalytic activity of the target interconvertible enzyme in response to stimuli. This rate amplification potential increases with the number of cycles in the cascade. The extent of the rate amplification is further enhanced if the multicyclic cascade involved possesses a pyramidal relationship with respect to the concentrations of its interconvertible enzymes. Moreover, if the converter and interconvertible enzymes are topographically positioned close to each other, an even greater rate of response is possible. It has been shown that multicyclic cascades can respond to primary stimuli within the millisecond range. Experimentally, Danforth *et al.* (1962) demonstrated that phosphorylation of phosphorylase *b* in response to electrical stimulation of frog sartorius muscle at 30°C can be accomplished with a half-time of 700 ms.

Energy Consumption

As shown in Figs. 1 and 2, for each complete cycle of a covalent modification/demodification cascade, one equivalent of nucleoside triphosphate is consumed. The capacity of a cyclic cascade system to maintain a steady-state is dependent on a constant supply of metabolic energy (ATP in most cases) to drive the modification reactions. It should be pointed out that the concentration of ATP *in vivo* is maintained at fairly constant levels (4-5 mM) which are several orders of magnitude greater than the concentrations of the enzymes involved in the cascades. In the absence of adequate donor molecules, the interconvertible proteins would be converted completely to the unmodified forms. Thus, the constant ATP flux through the cyclic cascade is the energy required to maintain the operation of cyclic cascades *in vivo*.

The rate of ATP consumption is determined by all the kinetic parameters needed to describe the cyclic cascade (Stadtman & Chock, 1977*b;* Shacter *et al.*, 1984; Goldbeter & Koshland, 1987; also Chapter 12 in this book). To estimate the ATP turnover in a cyclic cascade *in vivo,* literature values were used to calculate the ATP consumption rate for the phosphorylation of hepatic pyruvate kinase and of skeletal muscle glycogen phosphorylase. The resultant values were compared to a reported ATP turnover rate of 2 M/min for liver (Lipmann, 1981). This reported high value is an error, however (Meinke *et al.*, 1986). The ATP turnover rate in mammalian cells was estimated to be about 1-10 mM/min (see

Goldbeter & Koshland, 1987). With this consideration, the amount of energy required for covalent interconversion of enzyme is not negligible. It is therefore not surprising that many of the cyclic cascades are operated in a transient manner in response to stimuli. In addition, synchronous control of the forward and reverse converter enzymes and lowering of the steady-state rate for the modification/ demodification reactions would also minimize energy consumption.

Covalent Interconversion versus Single Allosteric Control

Metabolic regulation by cyclic cascades involves both covalent modification of enzymes/ proteins and allosteric interactions between effectors and enzymes. In view of the complexity of reversible covalent modification systems, one may wonder whether allosteric interactions between metabolites and enzymes alone could produce the regulatory properties of cyclic cascades. For example, consider signal amplification. In order for it to be physiologically significant, the following conditions have to be met:

1. A minimal concentration of metabolites should be required to initiate the biological response(s) within a reasonable time frame;

2. The reaction(s) induced by the metabolites must be catalytic such that one effector can activate more than one target enzyme molecule.

The first condition can be fulfilled either by the cyclic cascade mechanism or by very tight binding between the allosteric effector and the target enzyme. Because the rate of effector binding is limited by the diffusion rate of the reactants, high affinity can be achieved only by slowing down the off-rate for the enzyme-bound effector. However, tight binding would reduce the temporal efficiency of the control process. It should be emphasized that in a cyclic cascade, only a small fraction of converter enzyme need be activated to obtain significant modification of the interconvertible enzyme, so tight binding of a metabolite to the converter enzyme is not essential for signal amplification. In order to achieve a catalytic effect with a simple allosteric model, the effector would first have to bind to the target enzyme, induce an active conformation, and then dissociate from the active enzyme which would have to remain in the active conformation. However, to make the system regulatable, the active enzyme would have to be able to relax back to its inactive form. This type of mechanism is thermodynamically unfavourable (D. Astumian and P. B. Chock, unpublished results). In addition, in the absence of catalytic intermediates, there will be no rate amplification. Furthermore, the capacity for allosteric interactions in cascade systems is significantly enhanced relative to a simple allosteric model because there are more proteins involved in a cyclic cascade. On the other hand, of course, the apparent cooperativity which provides the sensitivity observed in cyclic cascades can be obtained by allosteric interactions alone, particularly if the enzyme involved contains multiple subunits. Therefore, signal amplification and rate amplification cannot be easily achieved without invoking reversible covalent

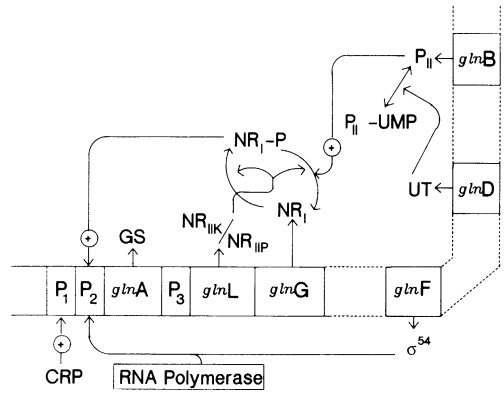


Figure 3. Relationship between various genes that regulate and code for glutamine synthetase and their respective products in regulating the transcription of glutamine synthetase: *glnF*, *glnG* and *glnL* are also known as *ntrA*, *ntr* and *ntrB* respectively. P_1, P_2, P_3 and CRP represent promoters, 1, 2, and 3 and cyclic AMP receptor protein, respectively. ⊕ indicates stimulation.

modification, while other regulatory properties of cyclic cascade can be accomplished but with less regulatory efficiency.

Bicyclic Cascade and Transcriptional Regulation of E. coli *Glutamine Synthetase*

The synthesis of glutamine synthetase is regulated in response to the availability of nitrogen and carbon source. *E. coli* cultures grown on limited nitrogen and excess glucose medium exhibit a high level of deadenylylated glutamine synthetase. In the presence of excess nitrogen in glucose-limited culture, glutamine synthetase level is repressed and fully adenylylated. The mechanisms for this transcriptional regulation are depicted in Figs. 1 and 3 (for review see Magasanik, 1982; Kustu *et al.*, 1986; Keener *et al.*, 1987). In this aspect, several gene products such as *glnG*, *glnL* (both are members of the *gln* operon) and an unlinked gene *glnF* are known to be involved (see Fig. 3). The *glnA* gene, which encodes glutamine synthetase, is transcribed from two promoters. The major nitrogen regulated promoter, P_2, lies closest to the structural information. To activate transcription from this promoter, both *glnF* and *glnG* products are required. A second promoter, P_1, lies upstream of the nitrogen-regulated promoter. Transcription from the upstream promoter is activated by the cyclic-AMP receptor protein bound to its ligand and does not require either the *glnF* or the *glnG* products. The product of *glnF* is a new σ subunit of RNA polymerase, σ^{54}, which confers a different promoter specificity on the core form of RNA polymerase from that conferred by the most abundant σ subunit, σ^{70} (Hirschman *et al.*, 1985; Ninfa *et al.*, 1987).

The linkage of the transcriptional regulation of glutamine synthetase to the bicyclic cascade is derived from the fact that the unmodified P_{II} facilitates the dephosphorylation of the *glnG* product, NR, which is catalysed by the *glnL* product, NR_{II}. The phosphorylated form of *glnG* product can stimulate transcription at the *glnA* promoter, P_2; while the unmodified form of NR_I is incapable of activating transcription. The *glnL* product, NR_{II}, appears to have both protein kinase and protein phosphatase activities for the interconversion of NR_I. Recently, Keener & Kustu (1988) showed that in *Salmonella typhimurium*

activation of transcription through P_2 promoter required RNA polymerase containing σ^{54} and phosphorylated NR_I. They also demonstrated that the kinase (NR_{IIK}) that catalyses this phosphorylation is a dimeric enzyme and it can undergo autophosphorylation. The maximum extent of phosphorylation is about 1 mol per mol of NR_I dimer. The phosphorylated NR_I is capable of undergoing autodephosphorylation. In the presence of NR_{II}, P_{II} and ATP, the rate of dephosphorylation of NR_I is enhanced by a factor of 4. Based on the data presented, it appears either that (i) NR_{II}-regulated dephosphorylation of NR_I phosphate can be derived from NR_{II} to function as an effector to enhance the autodephosphorylation of NR_I phosphate, or (ii) NR_{II} acts as a phosphatase. However, the overall results suggest that NR_{II} is a bifunctional enzyme and that the role of P_{II} and ATP is to exert a synergistic effect for the affinity between NR_I phosphate and NR_{II}.

In view of the observations that P_{II} inhibits the transcription of glutamine synthetase indirectly (see Fig. 3), the bicyclic cascade of glutamine synthetase, which initially was thought to modulate only the activity of the enzyme, is, in fact, tightly coupled to the transcriptional regulation of glutamine synthetase. As a consequence, fluctuation in intracellular concentrations of glutamine and α-ketoglutarate can be sensed by their effects on UT_u and UT_d activities, through which the signal is transmitted to both regulatory systems for adjusting both the concentration and activity of glutamine synthetase in the cells.

Concluding Remarks

The cyclic cascade model, derived mainly from data based upon detailed studies of glutamine synthetase, is applicable to all covalent interconvertible enzyme systems. It reveals many regulatory advantages such as signal amplification, rate amplification, sensitivity, and flexibility. This regulatory mechanism makes use of both covalent modification and allosteric interactions. By means of allosteric interactions with one or more enzymes, cyclic cascades can continuously monitor fluctuations in the concentrations of a multitude of metabolites and adjust the specific activities of the target enzymes in response to biological requirements. Thus, they serve as biological integrators. Although a cyclic cascade modulates the specific activity of the interconvertible enzyme smoothly and continuously over a wide range of conditions, it can under extreme physiological situations serve as an *on-off* switch to turn on or off the activity of an interconvertible enzyme. The energy for maintaining such an efficient regulatory mechanism is the consumption of ATP and other energy-rich donor molecules. In view of the unique properties of cyclic cascades, it is not surprising that a large number of key enzymes are regulated by this mechanism, both on their activities and their biosynthesis.

REFERENCES

Anderson, W. W. & Stadtman, E. R. (1970) *Biochem. Biophys. Res. Commun. 41,* 704-709
Brown, M. S., Segal, A. & Stadtman, E. R. (1971) *Proc. Natl. Acad. Sci. USA 68,* 2949-2953

Caban, C. E. & Ginsburg, A. (1976) *Biochemistry 15,* 1569-1580
Cárdenas, M. L. & Cornish-Bowden, A. (1989) *Biochem. J. 257,* 339-345
Chock, P. B. & Stadtman, E. R. (1977) *Proc. Natl. Acad. Sci. USA 74,* 2766-2770
Chock, P. B. & Stadtman, E. R. (1979) in *Modulation of Protein Function* (Atkinson, D. E. & Fox, C. F., eds.), Academic Press, New York, pp. 185-202
Chock, P. B., Rhee, S. G. & Stadtman, E. R. (1980) *Annu. Rev. Biochem. 49,* 813-843
Danforth, W. H., Helmreich, E. & Cori, C. F. (1962) *Proc. Natl. Acad. Sci. USA 48,* 1191-1199
El-Maghrabi, M. R., Claus, T. H., Pilkis, J., Fox, E. & Pilkis, S. J. (1982) *J. Biol. Chem. 257,* 7603-7607
Garcia, E. & Rhee, S. G. (1983) *J. Biol. Chem. 258,* 2246-2253
Goldbeter, A. & Koshland, D. E. Jr. (1981) *Proc. Natl. Acad. Sci. USA 78,* 6840-6844
Goldbeter, A. & Koshland, D. E. Jr. (1987) *J. Biol. Chem. 262,* 4460-4471
Hirschman, J., Wong, P. K., Sei, K., Keener, J. & Kustu, S. (1985) *Proc. Natl. Acad. Sci. USA 82,* 7525-7529
Jurgensen, S. R., Chock, P. B., Taylor, S., Vandenheede, J. R. & Merlevede, W. (1985) *Proc. Natl. Acad. Sci. USA 82,* 7565-7569
Keener, J. & Kustu, S. (1988) *Proc. Natl. Acad. Sci. USA 85,* 4976-4980
Keener, J., Wong, P., Popham, D., Wallis, J. & Kustu, S. (1987) in *RNA Polymerase and the Regulation of Transcription* (Reznikoff, W. S., Burgess, R. R., Dahlberg, J. E., Gross, C. A., Record, M. T., Jr. & Wickens, M. P., eds.), Elsevier Science Publishers, New York, pp. 159-175
Kustu, S., Sei, K. & Keener, J. (1986) in *Regulation of Gene Expression* (Booth, I. R. & Higgins, C. F., eds.), Cambridge University Press, Cambridge, Massachusetts, pp. 139-154
LaPorte, D. C. & Koshland, D. E., Jr. (1982) *Nature 300,* 458-460
Lipmann, F. (1981) *Curr. Topics Cell. Regn. 18,* 301-311
Magasanik, B. (1982) *Annu. Rev. Gen. 16,* 135-168
Mantel, M. & Holzer, H. (1970) *Proc. Natl. Acad. Sci. USA 65,* 660-667
Meinke, M. H., Bishop, J. S. & Edstrom, R. D. (1986) *Proc. Natl. Acad. Sci. USA 83,* 2865-2868
Mura, U., Chock, P. B. & Stadtman, E. R. (1981) *J. Biol. Chem. 256,* 13022-13029
Ninfa, A. J., Reitzer, L. J. & Magasanik, B. (1987) *Cell 50,* 1039-1046
Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun. 65,* 575-582
Rhee, S. G., Park, R., Chock, P. B. & Stadtman, E. R. (1978) *Proc. Natl. Acad. Sci. USA 75,* 3138-3142
Rhee, S. G., Bang, W. G., Koo, J. H., Min, K. H. & Park, S. C. (1988) in *Enzyme Dynamics and Regulation* (Chock, P. B., Huang, C. Y., Tsou, C. L. & Wang, J. H., eds.), Springer-Verlag, New York, pp. 136-145
Rhee, S. G., Chock, P. B. & Stadtman, E. R. (1989) *Adv. Enzymol. 62,* 37-92
Segal, A., Brown, M. A. & Stadtman, E. R. (1974) *Arch. Biochem. Biophys. 161,* 319-327
Senior, P. (1975) *J. Bacteriol. 132,* 407-418
Shacter, E., Chock, P. B. & Stadtman, E. R. (1984) *J. Biol. Chem. 259,* 12252-12259
Stadtman, E. R., Shapiro, B. M., Ginsburg, A., Kingdon, H. S. & Denton, M. D. (1968) *Brookhaven Symp. Biol. 21,* 378-396
Stadtman, E. R. & Ginsburg, A. (1974) *The Enzymes (3rd edn.) 10,* 755-807
Stadtman, E. R. & Chock, P. B. (1977) *Proc. Natl. Acad. Sci. USA 74,* 2761-2766
Stadtman, E. R. & Chock, P. B. (1978) *Curr. Topics Cell. Reg. 13,* 53-95
Stadtman, E. R. & Chock, P. B. (1979) in *The Neurosciences: Fourth Study Program* (Schmitt, F. O., ed.), MIT Press, Cambridge, Massachusetts, pp. 801-817
Stadtman, E. R., Chock, P. B. & Rhee, S. G. (1979) in *Modulation of Protein Function* (Atkinson, D. E. & Fox, C. F., eds.), Academic Press, New York, pp. 203-217
van Schaftingen, E., Davies, D. R. & Hers, H. G. (1982) *Eur. J. Biochem. 124,* 143-149

Chapter 14

Properties Needed for the Enzymes of an Interconvertible Cascade to Generate a Highly Sensitive Response

MARÍA LUZ CÁRDENAS and ATHEL CORNISH-BOWDEN

ONE OF THE important problems in biology is how to produce a sufficiently sensitive response to a signal. An essential point in metabolic control is thus the sensitivity in the response of a pathway to an effector. Consequently the understanding of the mechanisms that allow a high degree of sensitivity should constitute a major goal of any theory of metabolic control. Cooperativity in the response of an enzyme to an effector is undoubtedly an important mechanism, but it appears insufficient as the degree of cooperativity of enzymes is never very high (Hill coefficients less than 4 in nearly all cases). Thus even an effector that acts on a step with a flux control coefficient close to unity would only be able to switch on and off the pathway flux (say between 10% and 90% of full activity) if its concentration increases at least three-fold. Even a Hill coefficient as high as 6, rarely seen in nature, would only lower this ratio to two-fold. Furthermore, as flux control coefficients in reality are usually less than unity, the sensitivity of the pathway to the effector is decreased accordingly.

An alternative mechanism in nature that may permit a more sensitive response than is possible for the response of a single enzyme to an effector is the *interconvertible enzyme cascade* (reviewed in Stadtman & Chock, 1978; Chock *et al.*, 1980). However, this does not automatically provide a more sensitive response than direct interaction, as the degree of sensitivity is a function of the kinetic properties of the enzymes catalysing the interconversion (Cárdenas & Cornish-Bowden, 1989). Although there has been some investigation of the properties that the enzymes catalysing the conversion reactions must have if the cascade is to generate a very sensitive response (Goldbeter & Koshland, 1981, 1984; Fell & Small, 1986), there has been little emphasis on the fact that the cascade *per se* does not guarantee a more sensitive response to an effector than is possible for a single enzyme. This is not a trivial point, because cascades with the sort of properties often implicit

María Luz Cárdenas and Athel Cornish-Bowden ● Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

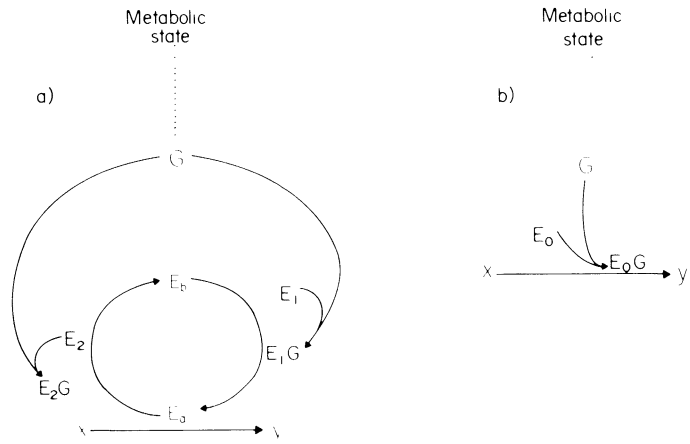


Figure 1. Activation of a target reaction by an effector. An effector G, whose concentration is a function of the metabolic state of the cell, stimulates the production of Y either directly or indirectly through a cascade. (*a*) *Monocyclic interconvertible cascade.* A target enzyme catalysing the conversion from X to Y exists in a catalytically active state E_a and in an inactive state E_b. These two forms can be interconverted by the action of two modifier enzymes, E_1 and E_2. G has no direct effect on the target enzyme, but controls its activity indirectly by activating E_1 or inhibiting E_2, or both. The asymmetrical appearance of the scheme derives from (and emphasizes) the fact that the actions of G on the two enzymes E_1 and E_2 are different. (*b*) *Direct activation.* G acts as an allosteric activator of the target enzyme.

in discussions generate a less sensitive response to effectors than the same effectors might have if they acted directly on a single enzyme (Cárdenas & Cornish-Bowden, 1989). Here we shall develop briefly these ideas and we shall apply them to an experimental system.

Model of a Monocyclic Enzyme Cascade

Reversible covalent modification of proteins is a major mechanism of cellular regulation, in both prokaryotic and eukaryotic cells (reviews in Chock *et al.*, 1980; Cohen, 1982; Niemeyer & Cárdenas, 1986; also Chapter 13 in this book by Chock, Rhee and Stadtman). In accordance with cellular requirements, an interconvertible protein is shifted between covalently modified and unmodified forms by two converter enzymes that catalyse opposing reactions. Such systems, in which one enzyme modifies another enzyme or protein, have been defined as *cascade systems* (Stadtman, 1970). The term cascade is sometimes taken to imply the existence of two or more distinct interconvertible enzymes; Goldbeter & Koshland (1981, 1982, 1984, 1987), for example, refer to monocyclic systems not as cascades but as *covalent modification systems*. It is also arguable whether any reversible systems ought to be called cascades, even qualified by an adjective emphasizing the cyclic character, as they differ in important respects from classical irreversible cascades, such as that involved in blood clotting. Despite these reservations, we shall use in this chapter the terminology that is best established in the literature (Stadtman & Chock, 1977; Chock & Stadtman, 1977), in which cascades embrace all covalent modification systems, and the terms *monocyclic, bicyclic* and *multicyclic* define the number of distinct interconvertible enzymes that make up each one.

We have systematically examined the response of a monocyclic cascade to an allosteric effector G. This model is illustrated in Fig. 1, and consists of a single interconvertible enzyme existing in two states E_b and E_a with different degrees of catalytic activity, (E_a being the more active form and E_b the less active or completely inactive one), the interconversion reactions being catalysed by two modifier enzymes E_1 and E_2, one or both activated or inhibited by G. We have determined the kinetic parameters needed by such a system to generate a highly sensitive response. One can pose several questions in relation to the kinetic parameters of E_1 and E_2 and their effects on the sensitivity of the cascade to the effector G. One must consider, for example, the type of inhibition, the type of activation, the degree of saturation, etc. Some of these questions have been considered previously (as discussed by Goldbeter and Koshland in Chapter 12 of this book). In relation to the inhibition of E_2 by G, for example, one ought to consider whether maximum sensitivity is provided by competitive inhibition, affecting the apparent value of V/K_m, (a *specific effect*); by uncompetitive inhibition, affecting the apparent value of V (a *catalytic effect*) or by mixed inhibition, affecting both. The same type of questions apply to the activation of E_1. Another important point is the relationship between the concentrations of G bringing about the activation and the inhibition: ought both enzymes E_1 and E_2 to have similar affinities for the effector G, as intuition might suggest? Ought the effector to act on both modifier enzymes or it is sufficient to act on one? If it acts on one only, does it matter which one?

Quantifying the Sensitivity of a Response

We have used two indexes to express the sensitivity properties of the cascade, the *response coefficient* (Burns *et al.*, 1985) and the *cooperativity index* of Taketa & Pogell (1965). The former derives from an agreement by several groups to express the degree of sensitivity of a system property f to an external parameter g by the response coefficient defined as the partial derivative $\partial \ln f/\partial \ln g$ and symbolized by R_g^f (Burns *et al.*, 1985; Kacser & Porteous, 1987; see also numerous chapters in this book, especially Chapter 3 by Porteous). It thus expresses the fractional response of an output variable f to a fractional change in an input variable g. For simplicity we shall consider the activity of the target enzyme as the output variable, effectively regarding the target reaction from X to Y as a one-step pathway. This is, of course, an oversimplification, and in reality the response coefficient for the flux will need to be adjusted in accordance with the flux control coefficient of the target enzyme (Fell & Small, 1986; Kacser & Porteous, 1987).

The cooperativity index of Taketa & Pogell (1965) corresponds to the ratio of g values needed for obtaining 10% and 90% of the maximal possible value of f. However, as it is useful to restrict the term cooperativity to the behaviour of a single protein, we shall use the term *sensitivity* for the corresponding property of a multienzyme system, which can occur in such a system even if no individual enzyme in it displays cooperativity. Accordingly, we shall use the name *sensitivity index* for the index of Taketa & Pogell, with the symbol r_G. In this context we note that the use by Goldbeter & Koshland (1981, 1984, 1987) of the term "response coefficient" and a symbol similar to R_g^f for the index of Taketa & Pogell conflicts with the agreed terminology (Burns *et al.*, 1985).

Modifier Enzymes Subject to Specificity Effects only

A monocyclic cascade may produce less sensitivity to an effector than one would have with direct interaction between effector and target reaction, and indeed must do so if one makes the simplest assumptions. Suppose that the modifier enzymes are susceptible only to effects on the apparent values of their specificity constants; that both have the same limiting rate V; and that E_1 has no activity when G is not bound to it (E_1 inactive, E_1G active) and E_2 has no activity when G is bound (E_2 active, E_2G inactive). The crucial assumption here is the restriction to specificity effects (Cárdenas & Cornish-Bowden, 1989); the others just simplify the analysis. With all these assumptions the rates v_1 (E_b to E_a) and v_2 (E_a to E_b) of the interconversion reactions in Fig. 1, catalysed by E_1G and E_2 respectively, are given by:

$$v_1 = \frac{Ve_b}{K'_{m1}(1 + K_a/g) + e_b} \tag{1}$$

$$v_2 = \frac{Ve_a}{K_{m2}(1 + g/K_i) + e_a} \tag{2}$$

where g is the concentration of G, and e_a and e_b are the total concentrations of E_a and E_b respectively. The specific activation constant of E_1 is K_a and the specific (competitive) inhibition constant of E_2 is K_i. In addition K'_{m1} and K_{m2} are the Michaelis constants for fully activated E_1 and uninhibited E_2 respectively (here and elsewhere in this paper primes are used for parameters that refer to enzyme forms with G bound).

The fraction f of target enzyme in the active state will be as follows:

$$f = \frac{e_a}{e_a + e_b} = \frac{1}{1 + \dfrac{K'_{m1}(1 + K_a/g)}{K_{m2}(1 + g/K_i}} \tag{3}$$

and we shall examine the effect that G has on this fraction via the cascade with the effect that it might have if it acted directly on the target enzyme. Thus we have examined the response coefficient in both cases. If G acted directly as a specific activator of a target enzyme E_o, catalysing the reaction X to Y, without the intermediacy of a cascade, the rate v of the reaction catalysed by E_0 at a concentration x of its substrate X would be given by eqn. (4).

$$v = \frac{V'_o x}{K'_{mo}(1 + K_{ao}/g) + x} \tag{4}$$

in which V'_o, K'_{mo} and K_{ao} are constants. Partial differentiation of eqn. (4) with respect to g shows that the response coefficient has a value that approaches 1 at low values of g:

$$R_g^v = \frac{\partial \ln v}{\partial \ln g} = \frac{1}{1 + (g/K_{ao})(1 + x/K'_{mo})} \tag{5}$$

In the case of the cascade the response coefficient is as follows:

$$R_g^f = \frac{\partial \ln f}{\partial \ln g} = \frac{\dfrac{g}{K_i + g} + \dfrac{K_a}{K_a + g}}{1 + \dfrac{K_{m2}(1 + g/K_i)}{K'_{m1}(1 + K_a/g)}} \tag{6}$$

In this case the response coefficient cannot exceed 2. Thus, in the numerator of eqn. (6) the first term represents the effect of G on E_1 and the second represents its effect on E_2: each has a limiting value of 1.0, which means that the limiting value of the numerator is 2.0. On the other hand, the denominator will be always bigger than 1.0, meaning that in optimal conditions the response coefficient cannot exceed 2 for a monocyclic cascade with the specified properties. Moreover, it will only be more than 1.0 if G affects *both* modifier enzymes and not necessarily even then; if it affects only one of these enzymes, G will always produce a less sensitive response than one would have with direct interaction. To obtain a value bigger than 1, not only must both enzymes be affected, but the activation constant K_a must be large compared with the inhibition constant, i.e. E_2 must be capable of being strongly inhibited by G at concentrations where the activation of E_1 is still slight; and it is also necessary that K'_{m1} be large compared with K_{m2}.

Introduction of cooperative interactions between G and the modifier enzymes does not alter the general conclusion that if only specificity effects operate a cascade does not necessarily produce a greater degree of sensitivity to an effector — and may produce less — than one could expect from direct interaction (with the same degree of cooperativity) between effector and target reaction (Cárdenas & Cornish-Bowden, 1989).

At the other extreme, there are conditions in which an enormous sensitivity, almost infinite for practical purposes, can be obtained with the monocyclic cascade. This will be seen in the next section.

General Model: Catalytic and Specific Effects

A very general model having the following characteristics has been analysed:

1. The two modifier enzymes in the cascade may have different limiting velocities ($V'_1 \neq V_2$).

2. The effector G may modify the apparent values of catalytic as well as specificity constants; i.e. in the case of inhibition it may have uncompetitive as well as competitive effects, and similarly for activation.

3. The inhibition and the activation are not necessarily complete, but can be hyperbolic, i.e. the enzymes may have some activity in the absence of activator or when saturated with inhibitor. In other words the enzyme species E_1 and E_2G can be partially active.

Some time ago, one of us studied the effect of an uncompetitive inhibitor on fluxes and concentrations within a pathway. It turned out that although for isolated enzymes there is not much difference between the effects of competitive and uncompetitive inhibitors under the

ordinary conditions of enzyme assay *in vitro,* the difference can become infinite in the extreme case of a constant flux and, in any case extremely large in the more realistic case in which both fluxes and concentrations can vary in response to the concentration of an external inhibitor (Cornish-Bowden, 1986). These drastic consequences of catalytic effects on fluxes led us to consider catalytic effects in cascades. Goldbeter & Koshland (1984) had earlier reported that classical noncompetitive inhibition of E_2 by the effector gave a higher sensitivity than just competitive inhibition, an observation that suggests also that one ought to analyse the importance of catalytic effects. As we shall see, addition of catalytic effects to the model is crucial for the production of high sensitivity. On the other hand, the other characteristics (unequal limiting velocities and hyperbolic effects) do not have major consequences, but they make the model more realistic.

With this general model, eqns. (1-2) for the rate of the two modification reactions must be replaced by eqns. (7-8):

$$v_1 = \frac{(V_1' + V_1 K_{G1}'/g)e_b}{K_{m1}'(1 + K_{G1}/g) + e_b(1 + K_{G1}'/g)} \tag{7}$$

$$v_2 = \frac{(V_2 + V_2' g/K_{G2}')e_a}{K_{m2}(1 + g/K_{G2}') + e_a(1 + g/K_{G2}')} \tag{8}$$

Unprimed symbols V and K_m refer to the limiting rates and Michaelis constants respectively of E_1 or E_2 (according to the numerical subscript) in the absence of effector G, whereas the corresponding primed symbols refer to the enzyme with effector bound to it. The effector constants K_{G1}, K_{G2}, K_{G1}' and K_{G2}' refer to dissociation of G from E_1G, E_2G, E_1E_bG and E_2E_aG respectively. The general model generates more complex equations that bring the model out of the range of simple algebraic analysis, and so instead of estimating the sensitivity through the response coefficient we studied the sensitivity by numerical simulation, looking for the parameters that will give the smallest possible value of the sensitivity index r_G. As eqns. (7-8) contain three concentrations and ten parameters, implying exploration in 13 dimensions, we reduced the dimensionality to nine by considering appropriate ratios of parameters, and by considering not the whole range of f values, but the two values 0.1 and 0.9, i.e.the values that define the variation of G concentration needed to vary the target reaction from a level of low activity, 10% of the limit, to one of high activity, 90% of the limit.

Conditions Necessary for Very High Sensitivity

Details of the dimensionless parameters and the numerical simulation can be found in Cárdenas & Cornish-Bowden (1989). A very important point is that a value of r_G as small as 1.0053 was found by the simulation, although the parameters allowed for the individual enzymes were highly constrained to be well within the range of the known behaviour of enzymes. Such a small value of r_G corresponds in practical terms to virtually infinite

sensitivity: an increase in only 0.5% of effector concentration is sufficient to bring the proportion of the target enzyme in the active state from 10% to 90%. This corresponds to a Hill coefficient of around 800 (calculated as $\log 81/\log 1.0053$).

The importance of maintaining each ratio of parameters at or near its best value was examined and, the dependence of the cooperativity index r_G on the individual parameter ratios was established. If all the other parameters are optimized the ratio V_2/V_1' has a barely perceptible effect[1] on the value of r_G.

The sensitivity implied by the value of r_G of 1.0053 is too enormous to be of any real physiological value, and might well be deleterious as it would generate great problems of instability. We have therefore estimated the less stringent conditions that will give a value of r_G lower than 1.55, corresponding approximately to a Hill coefficient of 10 — still very large by the standard of cooperativity observed with individual enzymes. The important point is to emphasize that in principle a monocyclic cascade has the potentiality for generating as much sensitivity as the system requires, and accordingly may constitute an adequate mechanism for switching pathways on and off. Equally, however, cascades not only have the potential to function as on/off mechanisms for metabolic pathways that require such switching, but also for allowing the fractional activities of the interconvertible enzymes to be varied progressively over a wide range, as emphasized by Chock, Rhee and Stadtman in Chapter 13 of this book: this appeared to be the case of the adenylylation of glutamine synthetase (Segal *et al.*, 1974) and the phosphorylation of the mammalian pyruvate dehydrogenase complex (Pettit *et al.*, 1975). What will happen in each particular case depends on the kinetic parameters of the converter enzymes and of the effectors involved.

Summary of the Conditions for High Sensitivity

In general the conditions for obtaining a very high sensitivity are as follows:

1. *Catalytic rather than specific effects*. The ratio of catalytic and specific activation constants for E_1, K'_{G1}/K_{G1}, must be as large as possible (at least 0.055 for obtaining a Hill coefficient of 10) and likewise the ratio of catalytic ("uncompetitive") and specific ("competitive") inhibition constants for E_2, K'_{G2}/K_{G2}, must be as small as possible, no greater than 17.5. Note that the model can tolerate substantial degrees of specific activation of E_1 or inhibition of E_2 (0.055 is not particularly large nor 17.5 particularly small), but it is better if the catalytic components predominate. This is an important point because even catalytic components appear trivial or pass unnoticed in studies of the isolated modifier enzymes may be crucial for generating an adequate response.

[1]The apparent conflict between this result and that reported by Goldbeter and Koshland in Chapter 12 is an artifact of different definitions of V: here we refer to actual limiting rates, and our observation that the sensitivity of the system hardly depends at all on the values of V'_1 and V_2 simply means that any change in these parameters can be compensated almost fully by an appropriate change in effector concentration. By contrast, Goldbeter and Koshland use V for the apparent limiting rates, i.e. parameters that already take account of effector concentration.

2. *Activation weaker than inhibition.* The ratio of the mean activation constant of E_1 and mean inhibition constant of E_2 must be as large as possible. Thus the mean activation constant divided by the mean inhibition constant, $\frac{1}{4}(K_{G1} + K'_{G1})[(1/K_{G2}) + (1/K'_{G2})]$, must be no less than 0.01 for obtaining a Hill coefficient of about 10. (The harmonic mean of inhibition constants has been used in this parameter because it gives a more realistic measure of "average" behaviour than the arithmetic mean, whereas the reverse is true for activation). Ideally E_1 should not be appreciably activated at the effector concentrations at which E_2 begins to be appreciably inhibited, though a large departure from this ideal can be tolerated. This condition is further analysed afterwards.

3. *Modifier enzymes near saturation.* The scaled Michaelis constants for fully activated E_1, $K'_{m1}/(e_a + e_b)$, and for fully uninhibited E_2, $K_{m2}/(e_a + e_b)$, must both be as small as possible, and in any event less than 1.75, for obtaining a Hill coefficient of 10, i.e. both modifier enzymes should operate at more than 36% saturation [$0.36 = 1/(1 + 1.75)$]. This agrees with the idea of "zero-order ultrasensitivity" emphasized by Goldbeter & Koshland (1981, 1982, 1984; also Chapter 12 of this book), though the term *zero-order* normally implies a state rather closer to saturation than 36%.

Non-Linear Inhibition or Activation

If all the other parameters are optimized it is of little importance whether the activation of E_1 and inhibition of E_2 are "linear". However, non-linearities in the activation and inhibition greatly decrease the tolerance for the ratio V_2/V'_1. Thus, if unactivated E_1 and maximally inhibited E_2 have negligible activity, i.e. $V'_2/V_2 = 0.01$, the ratio V_2/V'_1 has little effect on the degree of sensitivity. On the other hand, if $V_1/V'_1 = 0.01$, i.e., if unactivated E_1 has 1% activity, which in practice might not be detected in experiments with the isolated enzyme, decreasing V_2/V'_1 below 0.03 causes a steep decrease in sensitivity[2]. The bigger the magnitude of the non-linearity, the bigger will be the effect of variations in the ratio V_2/V'_1 on the sensitivity. For further details see Cárdenas & Cornish-Bowden (1989).

Effector Modifying only one Enzyme

As we discussed earlier, a monocyclic cascade in which the modifier enzymes are subject only to specific effects, and in which the effector acts on only one of them, will always produce a less sensitive response than one would have with direct interaction. In contrast, this is not the case if the effector produces catalytic effects. Thus a very high degree of sensitivity, with $r_G = 1.091$, corresponding to a Hill coefficient of about 50, is now possible even if G interacts with only one enzyme of the cascade. Although the same minimal value

[2]In Cárdenas & Cornish-Bowden (1989) there is an typographical error such that this condition is given as $V'_1/V_1 = 0.01$ instead of $V_1/V'_1 = 0.01$.

of r_G is obtained whichever of the two enzymes responds to G, the effect on the target enzyme is not equivalent in the two cases. Thus the two functions representing the dependence on g of the fraction of target enzyme in the active state (e_a) generate curves that are highly unsymmetrical. Either curve may be transformed into the other by translation and rotation through 180° about the half-conversion point. In the case where the effector only inhibits the enzyme E_2 (no-activation curve) the curve is very steep at low values of e_a and much less steep at high values, whereas the opposite is true of the curve where there is no inhibition and G activates the enzyme E_1 (no-inhibition curve). Further details are in Cárdenas & Cornish-Bowden (1989). So, although the minimum value of r_G is the same in both cases, to increase e_a 10-fold from 5 to 50% requires an increase in g of only 1.3% for the no-activation curve, whereas it requires an 18% increase for the no-inhibition curve. Consequently, if only one of the converter enzymes is affected by the effector, the inhibition of E_2 is much more important physiologically than the activation of E_1, as it allows a larger response coefficient at low concentrations of the target enzyme in active state. Thus if only one enzyme is affected in a real system, most probably this will be the one that is inhibited.

Activation Weaker than Inhibition

As was mentioned earlier, one of the conditions for obtaining a very high sensitivity is that the activation ought to be weaker than the inhibition. This point deserves further discussion, as it is a very important one, possibly the most important result obtained in this study. Fig. 2 illustrates this condition. It can be seen that the concentration of G for producing 50% inhibition of the modifier enzyme E_2 is much lower than that for 50% activation of E_1. Thus the corresponding curves of the dependence of the fraction of E_1 and E_2 in the active state on the concentration of G, are widely apart. This result is striking, as intuitively one would expect that the concentration of G that produces a significant inhibition of one would also produce significant activation of the other; Fig. 2, however, shows that this expectation is

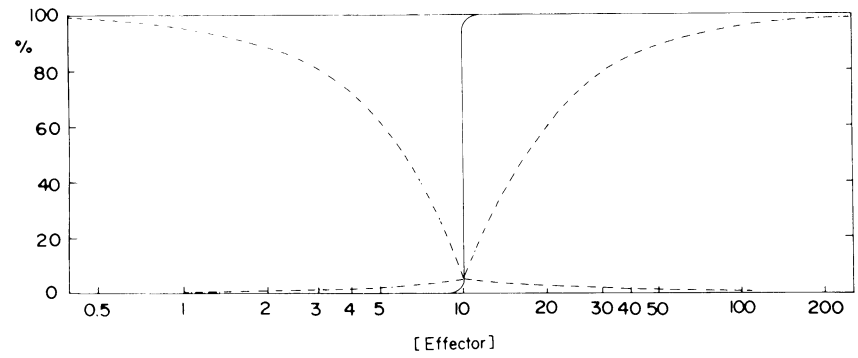


Figure 2. Proportions of the different enzymes of the cascade in the active state as a function of effector concentration. The simulation was made with the more general model assuming the optimum value for each ratio of parameters. The three curves show the fractions of modifier enzyme E_1 (· – · – · –), modifier enzyme E_2 (– – –), and target enzyme E_a (——) in the active state.

not true if the system is to respond with high sensitivity to G. Furthermore, the separation in the two curves has an important experimental implication: before deciding that G has no effect on E_1, one has to explore a wide range of concentrations, and not simply dismiss the activation of E_1 as non-existent or physiologically unimportant on the basis that there is no activation of E_1 at concentrations where the other converter enzyme is greatly inhibited. In the case of a bicyclic cascade Goldbeter & Koshland (1984) mention that the multi-step effect is optimized when the constant for activation of the forward step (E_1) is greater than the constant for inhibition of the reverse step (E_2), which increases the generality of our conclusion.

Another important point to notice in Fig. 2 is that the steep response of the target enzyme is produced in a range of concentrations of G where there is very little activity of E_1 and E_2, that is, the fraction of interconvertible enzyme in the active state increases dramatically at an effector concentration in which both converter enzymes are almost without activity. In this context it is interesting to note that in the case of a bicyclic cascade the fraction of the active form of the interconvertible enzyme of the second cycle increases abruptly at an effector concentration at which the fraction of the interconvertible enzyme of the first cycle (equivalent to E_1 in our symbols) in an active form is also very small (Goldbeter & Koshland, 1984; see also Fig. 3 of Chapter 12 in this book).

Application to an Experimental System: the Effect of Okadaic Acid

Muscle contraction provides an interesting experimental system for testing the ideas about a monocyclic cascade developed above. The degree of tension of the muscle fibre depends on the degree of phosphorylation of the light chains of myosin, which can exist in two states, phosphorylated and non-phosphorylated, the phosphorylation being catalysed by a protein kinase and the dephosphorylation being catalysed by a protein phosphatase. Thus any factor that stimulates the kinase or inhibits the phosphatase will stimulate muscle contraction.

In recent years it has been found that the toxin okadaic acid produces strong contractile effects on vascular and intestinal smooth muscles (Shibata *et al.*, 1982; Takai *et al.*, 1987; Bialojan *et al.*, 1988) and on heart muscle (Kodama *et al.*, 1986). Okadaic acid ($C_{44}H_{66}O_{13}$) is a polyether derivative of a monocarboxylic fatty acid that has been isolated from black marine sponges (Tachibana *et al.*, 1981); it has been very well studied because it appears to be the causative agent of diarrhoeic shell-fish poisoning (Murata *et al.*, 1982) and a very potent tumour promoter (Suganuma *et al.*, 1988). Because of its effect on muscle contraction it was suspected that it could affect the degree of phosphorylation of myosin by inhibiting the protein phosphatase, stimulating the protein kinase, or both. Takai *et al.*, (1987) in a very interesting work studied the effect of okadaic acid on fibre contraction and the effect on the protein phosphatase and the protein kinase, and found that it strongly inhibits myosin phosphatase and enhances tension development, whereas the kinase activity was unaffected. Fig. 3 illustrates some of its results. It can be seen that the maximal effect on fibre tension is obtained at an okadaic acid concentration of 5-10 μM. They consider that this value is in good agreement with the fact that the inhibition of the protein phosphatase is almost complete at that concentration. Although this is true, it is evident on examining Fig. 3

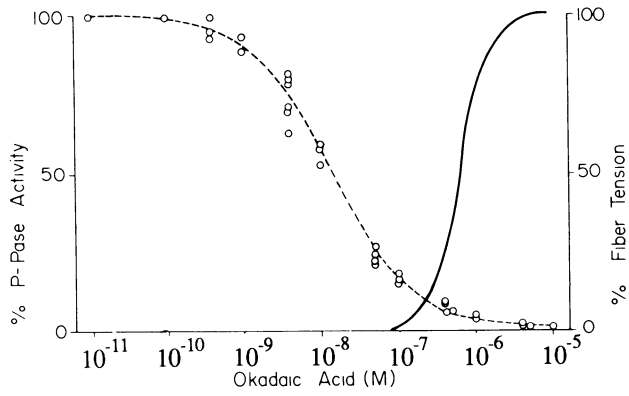


Figure 3. Effect of okadaic acid on the fibre tension (———) in taenia coli muscle, and on the protein phosphatase activity in the taenia coli extract (– – –). Various concentrations of okadaic acid were applied at a constant Ca^{2+} concentration of 1.5 μ M. Adapted from Takai *et al* (1987).

that there is very little fibre tension at concentrations of okadaic acid an order of magnitude lower (0.5 μM) or even smaller, although the phosphatase activity is still greatly inhibited. At first sight this result could be interpreted as indicating that the two processes are independent, but if one considers the results presented in Fig. 2 it becomes evident that what has been obtained by Takai and colleagues is what one ought to expect if the system is going to give a high sensitivity to okadaic acid. These authors also studied the effect of 5 μM okadaic acid on protein kinase at different calcium concentrations, and found no activating effect on the kinase although the phosphatase is almost completely inhibited at this concentration, concluding that okadaic acid does not affect protein kinase.

The response of fibre contraction to okadaic acid shows higher cooperativity than the response of the protein phosphatase inhibition, but the degree of cooperativity is not as high as one might expect. A reason for the rather low cooperativity may be that the calcium concentration present in the experiment allows a rather high protein kinase activity (about 50% of the maximal kinase activity). Our analysis suggests that at a lower calcium concentration, i.e. 0.7 mM, at which very little activity of the myosin light chain kinase is detected, a much higher sensitivity could be obtained. In guinea pig taenia coli fibre the calcium concentrations in conditions of relaxation is 0.1 mM (Brading, 1981), it is quite possible that when okadaic acid reaches the fibre *in vivo* the calcium concentration could be considerably lower than 1.5 mM, and consequently the system may be expected to respond with a higher sensitivity than that obtained by Takai *et al.* (1987). In fact Bialojan *et al.* (1988) show results that are compatible with the idea that at a lower calcium concentration the sensitivity in the response of the fibre to contraction by okadaic acid is higher.

This group has also studied the kinetics of the inhibition of different protein phosphatases by okadaic acid (Bialojan & Takai, 1988). From their careful and detailed kinetic study it was possible for us to calculate the inhibition constants. Thus, from the data shown in Table 2 of their paper, we have calculated the inhibition constants K_{G2} (specific effects) and K'_{G2} (catalytic effects) from the following equations:

$$V^{app} = V(1 + i/K'_i) \tag{9}$$

Table 1. Inhibition constants of different protein phosphatases for okadaic acid

Protein phosphatase	Substrate	Inhibition constants[1]		Idealized activation constants[2] of a putative protein kinase
		K_i, nM	K_i', nM	with $K_a = K_a'$, nM
Type 2A	Phosphorylated myosin light chain	0.93	4.9	160
Type 2A	Phosphorylase	1.09	1.09	110
Polycation-modulated	Phosphorylated myosin light chain	66	452	12000
Type I	Phosphorylated myosin light chain	200	200	20000

[1] The inhibition constants were calculated from Table 2 of Bialojan & Takai (1988) by means of eqns. (9-10).
[2] The activation constants were calculated assuming that they were equal to one another and to 100 times the harmonic mean of the inhibition constants (see text).

$$K_m^{app} = K_m \frac{1 + i/K_i}{1 + i/K_i'} \tag{10}$$

It can be seen in Table 1 that in all cases there is a mixed type of inhibition (catalytic as well as specific effects), in agreement with what one ought to expect, and that the ratio K_i'/K_i, corresponding to K_{C2}'/K_{C2} in relation to the model shown in Fig. 1, varies between 1.0 and 6.9: as this is less than 17.5 it is within the range that permits a Hill coefficient as high as 10 if the other conditions for high sensitivity are also met.

On the basis of these calculated inhibition constants we have estimated what the activation constants, assuming (for want of other information) them to be equal, i.e. that there are catalytic and specific effects of equal magnitude, would have to be to satisfy the condition that the activation ought to be weaker than the inhibition by a factor of about 100. The resulting values, shown in Table 1, are so large that it is quite possible that even if there was activation of protein kinase by okadaic acid this activation would not have been noticed because of insufficiently high concentrations of okadaic acid.

Recently it has been shown that okadaic acid rapidly stimulates protein phosphorylation not only in muscle cells but in several different types of intact cells and behaves like a specific protein phosphatase inhibitor in a variety of metabolic processes (Haystead *et al.*, 1989). On the other hand it appears to have no effect on protein kinases, at least at concentrations where the phosphatases are well inhibited. Consequently it has been assumed that the whole of the physiological effect of okadaic acid is due to inhibition of phosphatases. However, our analysis of the characteristics necessary for an interconvertible enzyme cascade to generate a highly sensitive response to an effector suggests that the potent effect of okadaic acid on phosphorylation could arise *because of*, not in spite of, a very weak capacity to activate the corresponding kinases.

Acknowledgements: We are grateful to Dr. Claude Souvignet for providing us with valuable information about okadaic acid.

REFERENCES

Bialojan, C. & Takai, A. (1988) *Biochem. J. 256,* 283-290

Bialojan, C., Ruegg, J. C., & Takai, A. (1988) *J. Physiol. (Lond.) 398,* 81-95

Brading, A. F. (1981) in *Smooth Muscle: an Assessment of Current Knowledge* (Bülbring, E., Brading, A. F., Jones, A. W. & Tomita, T., eds.) pp. 65-92, Edward Arnold, London

Burns, J. A., Cornish-Bowden, A., Groen, A. K., Heinrich, R., Kacser, H., Porteous, J. W., Rapoport, S.M., Rapoport, T. A., Stucki, J. W., Tager, J.M., Wanders, R. J. A. & Westerhoff, H. V. (1985) *Trends Biochem. Sci. 10,* 16

Cárdenas, M. L. & Cornish-Bowden, A. (1989) *Biochem. J. 257,* 339-345

Chock, P. B. & Stadtman, E. R. (1977) *Proc. Natl. Acad. Sci. USA 74,* 2766-2770

Chock, P. B., Rhee, S. G. & Stadtman, E. R. (1980) *Annu. Rev. Biochem. 49,* 813-843

Cohen, P. (1982) *Nature 296,* 613-620

Cornish-Bowden, A. (1986) *FEBS Lett. 203,* 3-6

Fell, D. A. & Small, J. R. (1986) *Biochem Soc. Trans. 14,* 623-624

Goldbeter, A. & Koshland, D. E., Jr. (1981) *Proc. Natl. Acad. Sci. USA 78,* 6840-6844

Goldbeter, A. & Koshland, D. E., Jr. (1982) *Q. Rev. Biophys. 15,* 555-591

Goldbeter, A. & Koshland, D. E., Jr. (1984) *J. Biol. Chem. 259,* 14441-14447

Goldbeter, A. & Koshland, D. E., Jr. (1987) *J. Biol. Chem. 262,* 4460-4471

Kacser, H. & Porteous, J. W. (1987) *Trends Biochem. Sci. 12,* 5-14

Kodama, I., Kondo, N. & Shibata, S. (1986) *J. Physiol. (Lond.) 378,* 359-373

Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen., P. & Hardie, D. G. (1989) *Nature 337,* 78-81

Murata, M., Shimatani, M., Sugitani, H., Oshima, Y. & Yasumoto. T. (1982) *Bull. Jpn. Soc. Sci. Fish. 48,* 549-552

Niemeyer, H. & Cárdenas, M. L. (1985) *Arch. Biol. Med. Exp. 18,* 331-358

Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Comm. 65,* 575-582

Segal, A., Brown, M. A. & Stadtman, E. R. (1974) *Arch. Biochem. Biophys. 161,* 319-327

Shibata, S., Ishida, Y., Kitano, H., Ohizumi, Y., Habon, J., Tsukitani, Y. & Kikuchi, H. (1982) *J. Pharmacol. Exp. Ther. 223,* 135-143

Stadtman, E. R. (1970) in *The Enzymes* (3rd edn., Boyer, P. D., ed.), vol. 1, pp 397-459. Academic Press, New York

Stadtman, E. R. & Chock, P. B. (1977) *Proc. Natl. Acad. Sci. U. S. A. 74,* 2761-2766

Stadtman, E. R. & Chock, P. B. (1978) *Curr. Topics Cell. Regul. 13,* 53-93

Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S. Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K. & Yamada, K. & Sugimura, T. (1988) *Proc. Natl. Acad. Sci. USA 85,* 1768-1771

Tachibana, Y., Scheuer, P. J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Giopichand, Y. & Schmitz, F. J. (1981) *J. Am. Chem. Soc. 103,* 2469-2471

Takai, A., Bialojan, C., Troschka, M. & Ruegg, J. C. (1987) *FEBS Lett. 217,* 81-84

Taketa, K. & Pogell, B. M. (1965) *J. Biol. Chem. 240,* 651-652

Chapter 15

Regulation of Muscle Glycogenolysis

RONALD D. EDSTROM, MARILYN H. MEINKE,
MARY E. GURNACK, DAVID M. STEINHORN,
XIURU YANG, RUI YANG and D. FENNELL EVANS

DURING MUSCLE contraction, a significant part of the energy used is derived from glycogen. The rate of glucose 1-phosphate production from glycogen is determined by the fraction of glycogen phosphorylase in the phosphorylated, active, *a* form (Madsen, 1986). The fractions of phosphorylase in the *a* and *b* (dephospho-, inactive) forms depend on the two enzymes, phosphorylase kinase and protein phosphatase-1, which in turn are influenced by other enzymes and regulatory factors, all of which form an integrated, multienzyme glycogenolytic complex (Fig. 1) (Hallenbeck & Walsh, 1986). In this report we describe three experimental approaches to the study of regulation of this complex.

Most previous investigations described kinetic and thermodynamic characteristics of isolated enzymes. By piecing together the individual reactions, the traditional cascade scheme was developed. With higher levels of organization this approach fails to describe adequately the observed behaviour (Hallenbeck & Walsh, 1986). Many of the properties of multienzyme systems are not evident by extrapolation from the parameters of the isolated enzymes and may be better explained by considering the entire set of coupled interactions (Shacter *et al.*, 1984; Srere, 1987; Cox & Edstrom, 1982; Cox *et al.*, 1987). On the other hand, due to the complexity of these enzyme systems in muscle, it is not possible to deduce the interactions of the enzymes through *in vivo* studies. In order to bridge that gap, we have developed an *in vitro* system that allows the phosphorylase *a–b* cycle to be studied as a steady-state system (Meinke *et al.*, 1986). This steady-state experimental system can be used to investigate the sensitivity to changes in signal level, response rate and energy required for regulation (Goldbeter & Koshland, 1987).

The glycogenolytic system has been described in isolated glycogen particles that contain all of the enzymes of glycogen degradation and synthesis (Dombradi *et al.*, 1984; Meyer *et al.*, 1970; Heilmeyer *et al.*, 1970; Haschke *et al.*, 1970). Phosphorylase kinase and protein

Ronald D. Edstrom, Marilyn Meinke, Mary E. Gurnack ● Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455, USA ◆ David M. Steinhorn ● Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota 55455, USA ◆ Xiuru Yang, Rui Yang, D. Fennell Evans ● Center for Interfacial Engineering, University of Minnesota, Minneapolis, Minnesota 55455, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

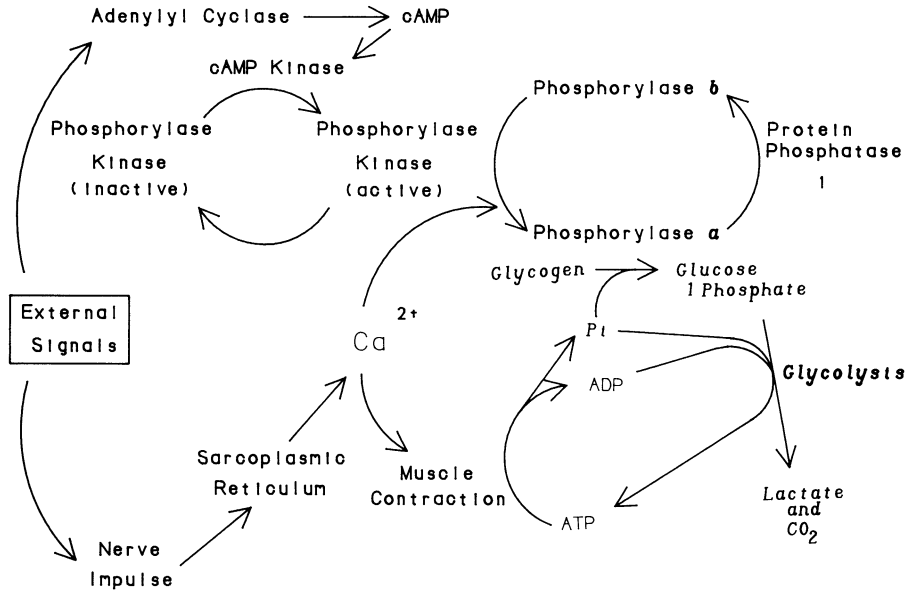


Figure 1. The regulation of muscle glycogenolysis is conducted by a complex network of signal pathways. The hormonal and neuronal stimuli are coordinated with the energy utilization of muscle contraction.

phosphatase-1 are organized within the isolated particles so as to allow their access to a specific set of protein components (Gruppuso & Brautigan, 1988). At that level of complexity, and with the contaminating activities present in the system isolated from muscle, it is not possible to control the multiple interactions at the individual enzyme level. To overcome these handicaps we have synthesized an artificial particle containing glycogen, phosphorylase and phosphorylase kinase. The other enzymes of glycogen metabolism and regulation can also be incorporated into the synthetic particles.

Some aspects of the architecture of the glycogenolytic complex can be deduced by indirect methods such as chemical crosslinking or analysis of the kinetics of glucose 1-phosphate release. Examination of the glycogenolytic complex by microscopic techniques provides an additional set of parameters to help describe the regulatory process. Direct observation of two of the enzymes of the glycogenolytic system has now been achieved by scanning tunnelling microscopy (Edstrom *et al.*, 1989). Structural features of the protein molecules can be discerned and their aggregation states observed.

Zero-order Ultransensitivity

Activation of phosphorylase kinase by cAMP-dependent protein kinase is not an isolated event *in vivo*. The combined actions of phosphorylase kinase, cAMP-dependent protein kinase, phosphorylase, phosphorylase phosphatase and glycogen represent the minimum

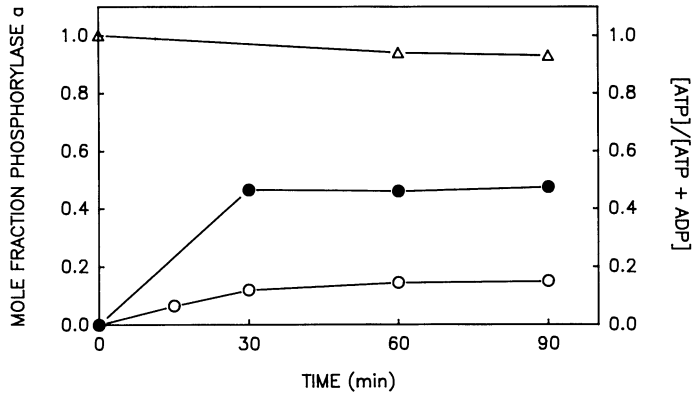


Figure 2. The mole fraction of phosphorylase in the *a* form increases during the incubation of phosphorylase *b* with phosphorylase kinase and protein phosphatase-1 in the presence of ATP. A steady state is reached when the accumulated phosphorylase *a* is hydrolysed by the phosphatase as rapidly as it is formed by the kinase. Phosphorylase concentrations were 1 μM (○) and 70μM (●). Maintenance of ATP level is also shown (△).

physiologically relevant system. Our experiments show that it is possible to assemble a system in which phosphorylase *a* and *b* are interconverted by phosphorylase kinase and phosphorylase phosphatase (Meinke *et al.*, 1986). The system operates in a cyclic, steady-state fashion where the level of phosphorylase *a* depends on the relative rates of the two converter enzymes. Their rates in turn are dependent on a variety of modulators such as cAMP-dependent protein kinase, calmodulin and Ca^{2+}.

Steady-state reactions were established with phosphorylase concentrations of 1, 20 and 70 μM. Phosphorylase kinase : phosphorylase phosphatase ratios were varied over a range of 0.1 to 10. Reactions were started with only phosphorylase *b* and were sampled for increasing phosphorylase *a*. Typical results are shown in Fig 2; in every case the steady steady state was reached within 60 min. Samples were analysed by high-performance liquid chromatography for adenine nucleotide content and tested at all times; only ADP and ATP were present, with the ATP/ADP ratio varying between 10 and 20. The absence of AMP precludes the possibility of spurious results due to activation of phosphorylase *b* by AMP.

The steady-state mole fraction of phosphorylase *a* is shown as a function of the normalized $V_{kinase}/V_{phosphatase}$ ratio in Fig 3. The lines drawn for each set of points are based on the parameters derived from the best fit of the data to the steady-state equation (Goldbeter & Koshland, 1981). The expression relates mole fraction of phosphorylase *a* to the ratio of the kinase and the phosphatase rates and the total phosphorylase concentration. Each data point represents the average of 3 to 9 separate steady-state incubations. The $V_{kinase}/V_{phosphatase}$ ratios have been normalized to cause all three sets of data to cross at the same point. K_m values derived from the data fitting are presented in Table 1 together with the K_m values from initial rate studies. Also shown in Table 1 are the response coefficients, R_v, defined as follows:

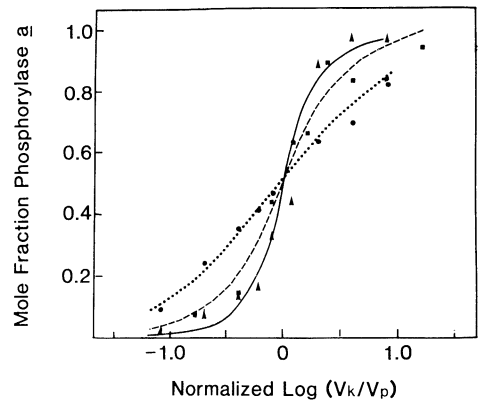


Figure 3. Demonstration of zero-order ultrasensitivity. As the concentration of phosphorylase increases from 1 (●) to 20 (■) to 70 μM (▲), the sensitivity of the steady-state cycle to changes in $V_{kinase}/V_{phosphatase}$ increases dramatically.

$$R_v = \frac{[V_{kinase}/V_{phosphatase}]_{90\%\ phosphorylase\ a}}{[V_{kinase}/V_{phosphatase}]_{10\%\ phosphorylase\ a}} = \frac{81(K_{kinase} + 0.1)(K_{phosphatase} + 0.1)}{(K_{kinase} + 0.9)(K_{phosphatase} + 0.9)}$$

The most responsive circumstance would require the smallest change in the $V_{kinase}/V_{phosphatase}$ ratio to go from 10 % to 90 % activated. For a maximally responsive system, R_v would approach 1.0, while for a system with no enhanced sensitivity R_v = 81. In the final column in Table 1 are listed the values of h', pseudo Hill coefficients, for the steady-state systems at the three substrate concentrations. Hill coefficients describe the sensitivity of a cooperative system to changes in effector concentration. A pseudo Hill coefficient can be calculated to describe the sensitivity of the zero-order system to changes in ratios of converter enzymes and it can be defined as: h' = log 81/log R_v. The pseudo Hill coefficient from the data at 70 μM phosphorylase (2.35) is comparable to true Hill coefficients found experimentally, such as for the binding of oxygen to haemoglobin with h = 2.8 (Edelstein, 1971), or the binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase with h = 2.3 (Kirschner *et al.*, 1971). Zero-order ultrasensitivity affords the phosphorylase system a degree of sensitivity similar to that found with classical cooperative enzymes.

Table 1. Kinetic parameters derived from steady-state reactions

Type of Experiment		K_m (μM) Kinase	K_m (μM) Phosphatase	R_v	h'
Steady-state[1]	at 1 μM	16	13	73	1.0
	at 20 μM	16	22	26	1.4
	at 70 μM	22	11	6.5	2.4
Initial Rate[1]		30	16	–	–

[1]The values of K_m obtained in the steady-state experiments were derived from fitting the experimental data to the steady-state equation (Goldbeter & Koshland, 1981). K_m values from initial rate studies were based on measuring reaction velocities as a function of substrate concentrations and fitting the data to the Michaelis-Menten equation.

Table 2. Comparison of Isolated and Synthetic Particles

	From muscle Weight %	From muscle Molar ratio	Synthetic Weight %	Synthetic Molar ratio
Sepharose	-	-	38	-
Concanavalin A	-	-	12	0.06
Glycogen	70	1	35	1
Phosphorylase	25	36	10	27
Phosphorylase kinase	5	0.6	5	1

Synthetic Glycogenolytic Particles

In skeletal muscle, phosphorylase and its two converter enzymes, phosphorylase kinase and protein phosphatase-1, are present in a glycogen particle complex. The other enzymes in these particles include branching and debranching activities, glycogen synthase and a Ca^{2+} dependent ATPase from sarcoplasmic reticulum. We have prepared a synthetic glycogen particle that binds phosphorylase and phosphorylase kinase and allows comparison of the kinetic behaviour of particle-bound enzymes with soluble forms.

Agarose beads (Sepharose 4B-200) were derivatized with concanavalin A and then treated with rabbit-liver glycogen, which bound to the concanavalin A. Phosphorylase and phosphorylase kinase both have specific binding sites for glycogen and could be bound to the glycogen impregnated beads. The composition of the synthetic particles is similar to glycogen particles isolated from skeletal muscle (Table 2). The diagram (Fig. 4) shows the preparation route for the particles. The two types shown at the bottom are both able to give a glycogenolytic response when exposed to $HOPO_3^{2-}$.

Soluble and particle-bound phosphorylase *b* behave differently towards the allosteric effector 5'-AMP (Fig 5). Phosphorylase *b* is much more sensitive to the nucleotide in the soluble state; it is fully activated by 0.2 mM 5'-AMP, whereas the particulate form of the enzyme requires at least 1 mM 5'-AMP for maximal activity, which was only 50% of that seen with soluble enzyme. Activation of phosphorylase *b* by phosphorylase kinase in solution and in enzyme-impregnated beads was compared at the same concentrations of both enzymes (Fig 6). The enzymes in the glycogen particle had in a lower activation rate for phosphorylase *b*. This must have been due to an impaired mobility of the glycogen bound phosphorylase kinase and its reduced accessibility to the similarly constrained phosphorylase *b*.

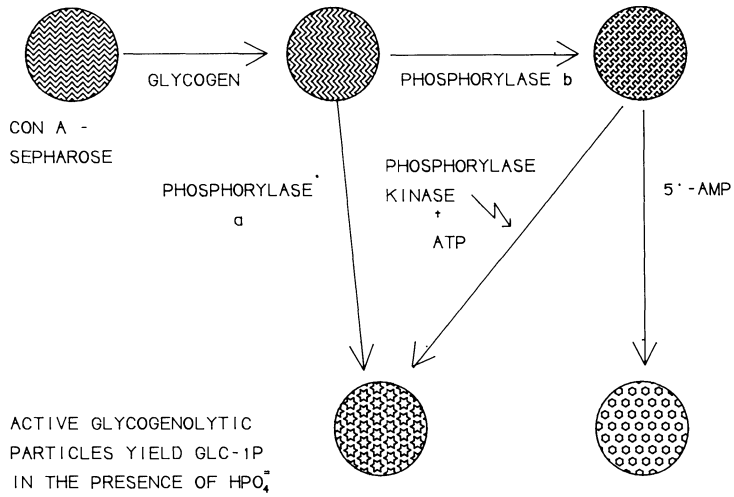


Figure 4. The active synthetic glycogenolytic particles can be obtained by three routes: directly with preformed phosphorylase *a* or by activation of phosphorylase *b* with either phosphorylase kinase or 5'-AMP.

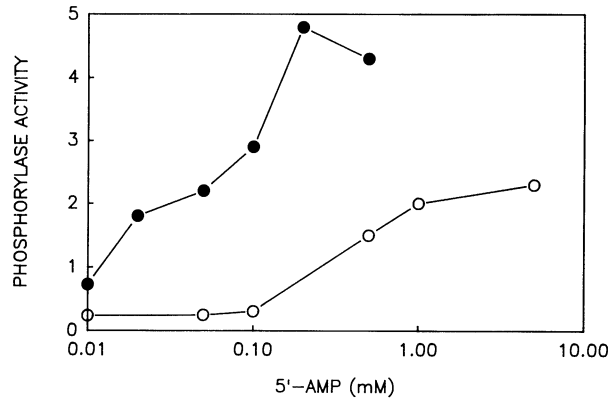


Figure 5. The soluble form of phosphorylase *b* (●) is much more susceptible to activation by 5'-AMP than when phosphorylase *b* is bound to the glycogen particles (○). Glucose 1-phosphate release was initiated by the addition of phosphate and measured by an assay coupled to NADH production.

The synthetic particles offer several experimental advantages over the glycogen-protein complexes isolated from muscle tissue (Meyer *et al.*, 1970; Entman *et al.*, 1980; Hallenbeck & Walsh, 1986). The synthetic particle system eliminates contaminating activities, particularly a very active Ca^{2+}-dependent ATPase that prevents studies requiring maintenance of significant ATP concentrations (Heilmeyer *et al.*, 1970). Another advantage of the synthetic particles is the complete freedom in choosing which components to add in their assembly. Kinetic studies require manipulation of the relative amounts of enzymes and regulatory factors. These synthetic particles are an approximate model for the muscle glycogen particles, which will allow studies of the interactions of the various enzymes and other elements of the signal transduction pathway in glycogenolysis.

Scanning Tunnelling Microscopy

Scanning tunnelling microscopy provides direct visualization of individual molecules of phosphorylase kinase and glycogen phosphorylase (Edstrom *et al.*, 1989). Phosphorylase kinase from rabbit skeletal muscle has an M_r of 1.3 x 10^6 with four types of subunits, each occurring in four copies – $(\alpha\beta\gamma\delta)_4$ (Pickett-Gies & Walsh, 1986). Electron microscopy and chemical crosslinking studies of the molecule indicate that it is a dimer of octamers $\alpha_2\beta_2\gamma_2\delta_2)_2$ (Fitzgerald & Carlson, 1984; Trempe *et al.*, 1986). Glycogen phosphorylase can

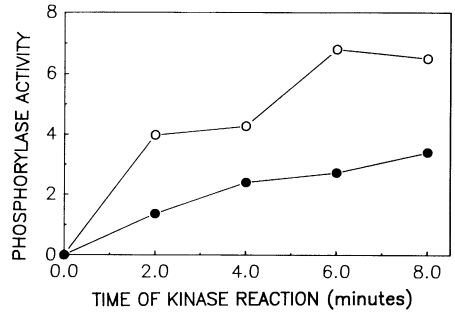


Figure 6. Activation of phosphorylase *b* (4 mg/mL) by phosphorylase kinase (3 μg/mL) was compared between the soluble system (○) and the particle bound reaction (●).

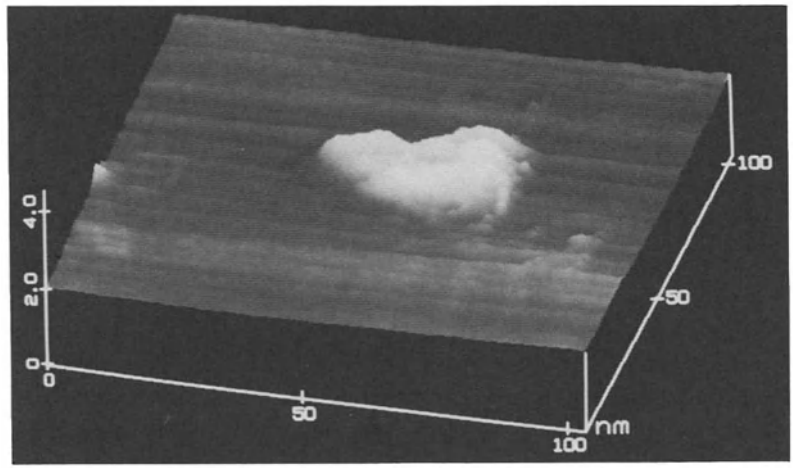


Figure 7. A single phosphorylase kinase molecule resting on the graphite surface. The Nanoscope II (Digital Instruments, Inc.) scanning tunnelling microscope was used.

occur as dimers or tetramers of identical subunits each having an M_r of 97 400. More highly aggregated forms also are known to exist (Chignell *et al.*, 1968). Its molecular structure has been resolved by X-ray crystallography to 0.19 nm with unit cell dimensions of 12.85 x 11.63 nm (Sprang *et al.*, 1988). The dimer has been reported to have the molecular dimensions 11.0 x 6.5 x 5.5 nm (Chignell *et al.*, 1968; Puchwein *et al.*, 1970).

In scanning tunnelling microscopy, a sample placed on an atomically flat graphite surface is scanned by a wire probe attached to a computer-driven ceramic piezo-electric device (Hansma & Tersoff, 1987). A small voltage is maintained between the probe and the surface which results in a tunnelling current over the short distance between them (≈1 nm). As an object on the surface is encountered, an image is generated based on the vertical distance the probe moves to maintain a constant tunnelling current. A typical image of phosphorylase kinase is shown in Fig. 7. The butterfly-shaped molecule is approximately 24 nm across the "wings" and about 21 nm in the front-to-back dimension. The central portion of the molecule between the lobes appears to be depressed with respect to the outer areas. Glycogen phosphorylase molecules have also been examined by scanning tunnelling microscopy and found in a variety of aggregation states, including dimers and tetramers as well as both large condensed and linear aggregates. A small portion of a 710 nm linear aggregate is shown in Fig 8. The width of the chain was 10.7 nm and it had a periodic pattern along the chain with a repeat distance of 5.6 ± 0.33 nm. Details of protein shape and subunit organization are clearly visible.

The images of phosphorylase *b* are consistent with the measurements based on X-ray crystallography, X-ray scattering and electron microscopy (Chignell *et al.*, 1968; Puchwein *et al.*, 1970). The ability to visualize protein molecules such as phosphorylase kinase and phosphorylase *b* and its aggregates suggest that scanning tunnelling microscopy can make a major contribution to protein chemistry and our understanding of multi-protein complexes.

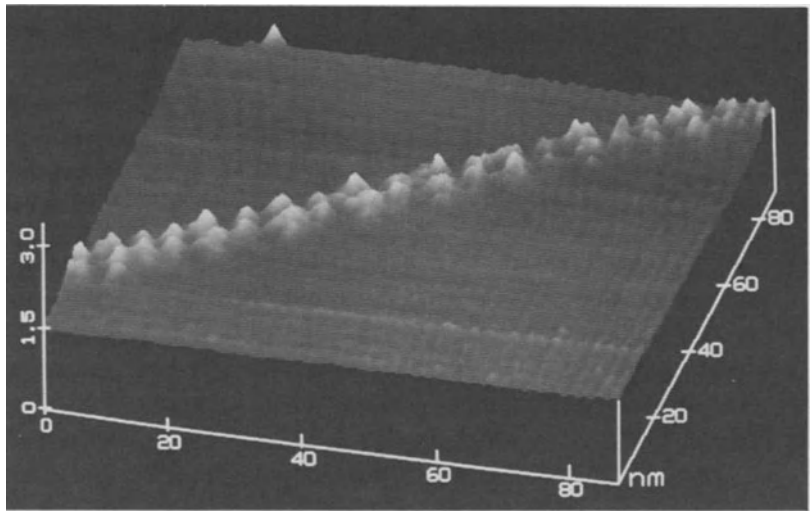


Figure 8. A chain of phosphorylase *b* molecules. The chain was 11 nm wide and Fourier analysis indicated a major periodicity of 5.7 nm.

Discussion

The three experimental approaches described here are designed to investigate the regulatory network of glycogenolysis as a system. Studying the separate enzymes and effectors of a regulatory system will not provide sufficient information to describe the regulatory properties of the system. Nevertheless, it is only possible to study regulatory systems well when extensive information on the properties of the individual components is available, as is the case with glycogenolysis. Substantial theoretical work has begun on the relationships of regulatory cascades and networks (Stadtman & Chock, 1978; Goldbeter & Koshland, 1981; Cárdenas & Cornish-Bowden, 1989; see also Chapters 12-14 in this book). Those studies will provide the basis for construction of a set of models to be tested experimentally.

The amplification provided by zero-order ultrasensitivity must play an important role in muscle glycogenolysis. With K_m values near 20 μM for both the kinase and the phosphatase and a 70 μM bulk concentration of phosphorylase in muscle, the zero-order effect would be quite pronounced. In fact, in muscle, the phosphorylase is confined to the 2% of the tissue space occupied by glycogen particles. That compartmentation increases the local concentration of phosphorylase to 3.5 mM. At that concentration, zero-order ultrasensitivity would result in a pseudo Hill coefficient of 38, indicating that this regulatory step is much more sensitive than any allosterically controlled enzyme. Preliminary examination of some of the other steps in the glycogenolytic regulatory network show that such amplification is not to be expected throughout. This is not surprising, as there may not be much more than a ten-fold amplification of the system between the Ca^{2+} or cAMP input signals and the change in phosphorylase *a* (Chasiotis, 1983).

An additional consideration for studying regulatory systems of this sort is that many will

be found to be spatially organized systems. Certainly in the case of glycogenolysis most of the enzymes in the regulatory network are bound in the glycogen particle. Imaging of molecular complexes by scanning tunnelling microscopy will help us understand the architecture of these regulatory systems. Future theoretical work is needed to translate our present solution models into kinetic descriptions of enzymes in the solid or gel state. There are many challenges for both theoreticians and experimentalists in this new and interesting area of biochemistry.

Acknowledgements: This work was supported by the American Diabetes Association – Minnesota Affiliate, the University of Minnesota Graduate School, the Minnesota Medical Foundation and the National Science Foundation

REFERENCES

Chasiotis, D. (1983) *Acta Physiol. Scand. (Suppl) 68,* 1-110
Chignell, D. A., Gratzer, W. B. & Valentine, R. C. (1968) *J. Biol. Chem. 7,* 1082-1089
Cox, D. E. & Edstrom, R. D. (1982) *J. Biol. Chem. 257,* 12728-12733
Cox, D. E., Meinke, M. H. & Edstrom, R. D. (1987) *Arch. Biochem. Biophys. 259,* 350-362
Cárdenas, M. L. & Cornish-Bowden, A. (1989) *Biochem. J. 257,* 339-345
Dombradi, V., Gergely, P. & Bot, G. (1984) *Acta Biochim. Biophys. Acad. Sci. Hung. 19,* 193-201
Edelstein, S. J. (1971) *Nature 230,* 224-227
Edstrom, R. D., Meinke, M. H., Yang, X., Yang, R. & Evans, D. F. (1989) *Biochemistry 28,* 4939-4942
Entman, M. L., Keslensky, S. S., Chu, A. & Van Winkle, W. B. (1980) *J. Biol. Chem. 255,* 6245-6252
Fitzgerald, T. J. & Carlson, G. M. (1984) *J. Biol. Chem. 259,* 3266-3274
Goldbeter, A. & Koshland, D. E., Jr. (1981) *Proc. Natl. Acad. Sci. USA 78,* 6840-6844
Goldbeter, A. & Koshland, D. E., Jr. (1987) *J. Biol. Chem. 262,* 4460-4471
Gruppuso, P. A. & Brautigan, D. L. (1988) *Biochem. Int. 16,* 1027-1032
Hallenbeck, P. C. & Walsh, D. A. (1986) *J. Biol. Chem. 261,* 5442-5449
Hansma, P. K. & Tersoff, J. (1987) *J. Appl. Phys. 61,* R1-R23
Haschke, R. H., Heilmeyer, L. M. G., Jr., Meyer, F. & Fischer, E. H. (1970) *J. Biol. Chem. 245,* 6657-6663
Heilmeyer, L. M. G., Jr., Meyer, F., Haschke, R. H. & Fischer, E. H. (1970) *J. Biol. Chem. 245,* 6649-6656
Kirschner, K., Gallego, E., Schuster, I. & Goodall, D. (1971) *J. Molec. Biol. 58,* 29-50
Madsen, N. B. (1986) in *The Enzymes* Vol. 17 (Boyer, P. D. & Krebs, E. G., eds.), pp. 365-394, Academic Press, Orlando
Meinke, M. H., Bishop, J. S. & Edstrom, R. D. (1986) *Proc. Natl. Acad. Sci. USA 83,* 2865-2868
Meyer, F., Heilmeyer, L. M. G., Jr., Haschke, R. H. & Fischer, E. H. (1970) *J. Biol. Chem. 245,* 6642-6648
Pickett-Gies, C. A. & Walsh, D. A. (1986) in *The Enzymes*, Vol. 17 (Boyer, P. D. & Krebs, E. G., eds.), pp. 395-459, Academic Press, Orlando
Puchwein, G., Kratky, O., Golker, C. F. & Helmreich, E. (1970) *Biochemistry 9,* 4691-4698
Shacter, E., Chock, P. B. & Stadtman, E. R. (1984) *J. Biol. Chem. 259,* 12252-12259
Sprang, S. R., Acharya, K. R., Goldsmith, E. J., Stuart, D. I., Varvill, K., Fletterick, R. J., Madsen, N. B. & Johnson, L. N. (1988) *Nature 336,* 215-221
Srere, P. A. (1987) *Ann. Rev. Biochem. 56,* 21-56
Stadtman, E. R. & Chock, P. B. (1978) *Curr. Top. Cell. Regul. 13,* 53-95
Trempe, M. R., Carlson, G. M., Hainfeld, J. F., Furcinitti, P. S. & Wall, J. S. (1986) *J. Biol. Chem. 261,* 2882-2889

Part IV

METHODS OF APPLYING CONTROL ANALYSIS TO REAL SYSTEMS

Chapter 16

Methodology for Simulation of Metabolic Pathways and Calculation of Control Coefficients

PEDRO MENDES, JOÃO MONIZ BARRETO,
ANA VAZ GOMES and ANA PONCES FREIRE

THE MAIN advantage of metabolic control theory is that it is not necessary to consider enzyme kinetic mechanisms for an experimental analysis of real biochemical systems. Control coefficients can be directly determined by measuring the system response to a small perturbation of some external parameter. This can be accomplished without any knowledge of the kinetic model; the only requirement is that the system response may be measured.

If a system is to be simulated a model is needed that relates responses to perturbations. To be realistic this model should take account of the time dimension, either implicitly or explicitly. In both cases rate equations must be considered; the only difference is that in the latter case these rate equations are integrated whereas in the former they are assumed to be equal to a determined value that results in a set of algebraic equations. The integration of the rate equations provides the knowledge of the dynamic behaviour of the model.

Method

Control coefficients are determined experimentally making small perturbations on the system and measuring its response. In simulation, this approach can also be used but as it is error prone (small is not infinitesimal!) it is much better to do it from the elasticities using either the matrix method (Fell & Sauro, 1985; Sauro *et al.*, 1987; see also Chapter 9 by Fell, Sauro and Small in this book) or control-pattern analysis (Hofmeyr, 1989; see also Chapter 19 by Hofmeyr in this book). The elasticity coefficients could also be determined by the perturbation/response method, as in experiment, but again this carries some error; a

Pedro Mendes, João Moniz Barreto, Ana Vaz Gomes and Ana Ponces Freire ● Centro de Estudos de Bioquímica e Fisiologia Animal, Instituto Rocha Cabral, Calçada Bento da Rocha Cabral 14, 1200 Lisboa, Portugal

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

much better way is to use the local rate equations: elasticity functions are then obtained by differentiation.

At this point elasticity, control and combined response coefficients can all be calculated for sets of metabolite concentrations, which are the variables of the elasticity functions. As these coefficients are only defined for steady state, the concentrations must belong to a steady state of the model considered.

The determination of steady state concentrations must be based on a kinetic model and can be done by one of two procedures: a finite value for the steady state is considered such that the rate equations form a system of algebraic equations with the metabolite steady-state concentrations as solution. Otherwise initial values are attributed to the metabolite concentrations and the rate equations form a system of differential equations that can be integrated along the reaction time to obtain the metabolite steady state concentrations.

The kinetic models can take a macroscopic or microscopic form. If each reaction is taken as a whole the model is *macroscopic* and there are as many equations as there are metabolites (including source and sink). These equations should represent the properties of each reaction and could be, for example, of the Henri-Michaelis-Menten type. If the detailed molecular mechanism is considered the model is *microscopic* and the reaction intermediates are taken into account by the model. In microscopic models there are as many equations as chemical species, which can be a large number even for small systems. In both cases the mathematical model is a system of differential equations, generally non-linear. An example will be examined in the Discussion showing the differences between these approaches.

The external parameters have not yet been mentioned because they depend on which type of model is used. In microscopic models these will be concentrations of external effectors and enzymes, together with first-order and pseudo-first-order kinetic constants; in macroscopic models kinetic constants take a more elaborate form (such as V, K_m for hyperbolic enzymes). Parameters must be defined before any simulation is carried out.

With all the calculations already described some other useful physical quantities can be obtained, for instance flux, mass action ratios and transition times (Easterby, 1981; see also Chapter 23 by Easterby in this book).

Discussion

A major problem in simulating metabolic pathways lies in knowing the values of the elasticity coefficients. Simulation could be done varying the elasticity coefficients and calculating control coefficients. If this is done over a wide range of values the results may be informative but unrealistic for concrete models. The internal metabolite concentrations are constrained by the solution of a system of differential equations, and so are the elasticity coefficients. As the elasticities for a metabolite are functions of the same variable, its concentration, it seems very obscure to treat them as independent. Treating the elasticities as independent variables is acceptable if the aim is to study their effect on control coefficients. If one wants to simulate a model then this can no longer be an acceptable procedure. The objective of simulation is to correlate experimental results with some model and so it must

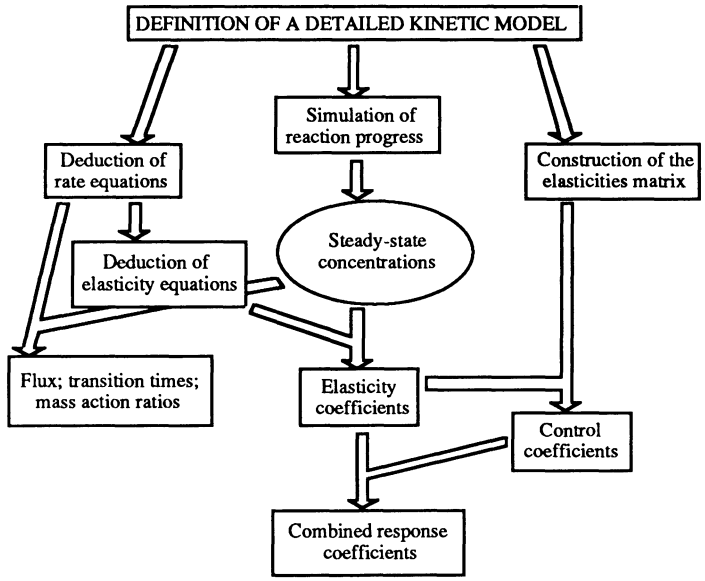
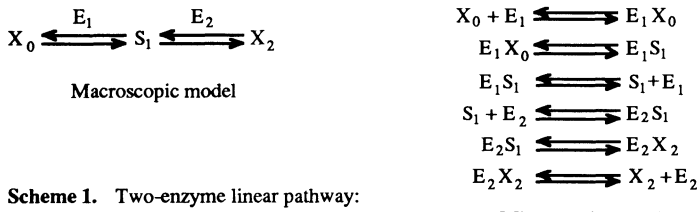


Figure 1. Schematic representation of methodology

be made through that model. Kacser & Burns (1979) have pointed out that a major advantage of metabolic control theory is that analysis can be made independently of kinetic models. This only applies to experimental approaches, as in simulation one is specifically concerned with a model. The interaction of simulation and experimental results can be very useful to the understanding of specific problems.

The central problem in the proposed methodology for simulation, which is illustrated schematically in Fig. 1, is the determination of steady-state concentrations. Different procedures have been described above. The integration of the differential equations along the reaction time seems to be the most advantageous as it provides a great deal of information. It provides not only the concentration vector but also allows one to follow the reaction progress. However some caution is appropriate, as this procedure involves two approximations: (*a*) the asymptotic nature of steady states makes them impossible to attain in finite time (although they can be approached arbitrarily closely); (*b*) the integration of differential equations (generally non-linear) is accomplished by numerical methods. The rate equations that are usually considered for biochemical reactions are non-linear and stiff. This is a technical problem that cannot be forgotten and specific numerical methods must be used. These can be the Gear method (probably the best) or Runge-Kutta semi-implicit methods (Kubicek & Marek, 1983; Hayashi & Sakamoto, 1986).

The choice between microscopic and macroscopic kinetic models should be made according to the pathway considered. Microscopic models should be chosen as they provide more information than macroscopic. However they may be almost impossible to use as the number of equations can be extremely large. Macroscopic models are then very useful as they are mathematically simpler. It is very important that the rate equations considered in macroscopic models describe the relevant properties of the real system. An example



Scheme 1. Two-enzyme linear pathway: Macroscopic and microscopic models.

showing the differences of micro and macroscopic kinetic models is considered. Scheme 1 shows a small two-enzyme linear pathway. In the macroscopic approach there are three differential equations (one for each metabolite) whereas in the microscopic there are nine (one for each chemical species). The differential equations describing the variation of S_1 with time would be as follows, first with a macroscopic approach:

$$\frac{\partial S_1}{\partial t} = \frac{\dfrac{V_1^f X_0}{K_{m_1}^f} - \dfrac{V_1^r S_1}{K_{m_1}^r}}{1 + \dfrac{X_0}{K_{m_1}^f} + \dfrac{S_1}{K_{m_1}^r}} - \frac{\dfrac{V_2^f S_1}{K_{m_2}^f} - \dfrac{V_2^r X_2}{K_{m_2}^r}}{1 + \dfrac{S_1}{K_{m_2}^f} + \dfrac{X_2}{K_{m_2}^r}}$$

and second with a microscopic approach:

$$\frac{\partial S_1}{\partial t} = k_{13}[E_1S_1] - k_{14}[E_1][S_1] - k_{21}[E_2][S_1] + k_{26}[E_2S_1]$$

If the macroscopic model were to be chosen, there would be no information about the enzyme-metabolite complexes but it would certainly be faster to solve the system of equations. On the other hand, if the microscopic model is to be used, then rate equations for the overall enzyme reactions must be derived so that the elasticity functions could be obtained by differentiation. The derivation of rate equations from the molecular mechanism could be done by the method of King & Altman (1956), or other. This example clearly shows the advantages and disadvantages of both approaches. A choice should be made according to the topology of the model.

REFERENCES

Easterby, J. S. (1981) *Biochem. J. 199,* 155-161

Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561

Hayashi, K. & Sakamoto, N. (1986) *Dynamic Analysis of Enzyme Systems,* pp. 59-88, Springer-Verlag, New York

Hofmeyr, J.-H. S. (1989) *Eur. J. Biochem. 186,* 343-354

Kacser, H. & Burns, J. A. (1979) *Biochem. Soc. Trans. 7,* 1149-1160

King, E. L. & Altman, C. (1956) *J. Phys. Chem. 60,* 1375-1378

Kubicek, M. & Marek, M. (1983) in *Computational Methods in Bifurcation Theory and Dissipative Structures* (Cabannes, M., Holt, M., Keller, H. B., Killen, J. & Orszag, S. A., eds.), pp. 87-90, Springer-Verlag, NewYork

Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221

Chapter 17

Regulatory Responses and Control Analysis: Assessment of the Relative Importance of Internal Effectors

HERBERT M. SAURO

WHAT DO WE MEAN by regulation? The term is not well defined and we use it perhaps a little too easily when we come to talk about metabolism and its "regulation". If we were to ask n biochemists we would probably get m (greater or less than n) answers, somewhat related, but different. As may be demonstrated by comparing the views expressed in different chapters of this book, there is no universally agreed definition nor, in consequence, any quantitative measure (however defined).

If we said that by regulation we meant some sort of response of metabolism to a change in an external influence then most people would probably agree. How the change comes about and whether there are different kinds of response, though, is another matter. I think part of the answer lies with our failure to ask definite questions that demand a quantitative approach. Given a regulatory response, i.e. given that there have been small changes to concentrations of substrates, effectors, products, etc., as a result of some external stimulus, can we assess the contributions that each of these changes makes to the the change in flux at a particular step? In order to try to answer such a question, we need a suitable quantitative framework, for example metabolic control analysis.

In control analysis, we have at our disposal three types of coefficients for examining metabolic pathways, *elasticities, control coefficients* and *response coefficients*. Porteous discusses these coefficients generally in Chapter 3 of this book. Here I shall consider them in the specific context of their role in answering the question that I have just posed.

The Elasticity Coefficient: $\varepsilon_S^v = \dfrac{\partial v}{\partial S} \cdot \dfrac{S}{v}$

The elasticity coefficient may be regarded as the "basic building block" of metabolic control analysis. It constitutes the property of an isolated enzyme responding to changes in the con-

Herbert M. Sauro ● Department of Genetics, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

centrations of its substrates, products, effectors or anything that might affect it (collectively termed *effectors*). It measures the response of the rate (the "local rate") of the reaction to changes in one of the potential effectors of the enzyme. With it there comes a very important concept, namely the idea of a *local rate* (*not* to be confused with the flux, which will be considered later). The local rate is the rate of the reaction of the isolated step with all effector concentrations held constant (clamped) at the values they have in the system. The local rate, v, is usually represented by one of the familiar rate expressions (such as the Michaelis equation or a more complex formulation) and may involve more than one molecular participant together with one or more parameters. In general,

$$v = f(E, S_1, S_2, \cdots, k_1, k_2, \cdots)$$

We shall consider a *change in the local rate,* brought about by a change in *one* of the effector concentrations or one of the parameters associated with the enzyme. It must be clear then that if a particular reaction is affected by, say, three different effectors (e.g. the substrate, product and an inhibitor) then we can define three different concentration elasticity coefficients, depending on which effector concentration we change. Additionally, the change in local rate can be brought about by a change in one of the parameters, say E, k_1, k_2, etc.).

The elasticity coefficients are dimensionless numbers and represent potential responses in the system. The larger in magnitude, then, the more sensitive is the local rate to actual changes in the effector concentration. Moreover, the value can be positive or negative. Could the elasticities be candidates for the measure of the regulatory importance of a step? As elasticities are local properties, whereas a regulatory response involves the whole system, the values of of individual elasticities would clearly not be suitable candidates. On the other hand we might feel that if a certain effector has a large elasticity for a particular step then that step might be more significant to the regulatory event than other steps. We will keep this last point in mind.

The Control Coefficients: $C_v^J = \dfrac{\partial J}{\partial v} \cdot \dfrac{v}{J}$ and $C_v^S = \dfrac{\partial S}{\partial v} \cdot \dfrac{v}{S}$

The control coefficients, in contrast to the elasticity coefficients, are true system properties. The indicate how the system flux, J, or a metabolite concentration, S, is affected by changes in the local rate of a particular reaction when the change is brought about by a parameter change. The system flux is equal to the local rate *in magnitude* when the latter is measured under the conditions prevailing in the system. Conceptually, however, the flux is quite different from the local rate because fluxes are determined by the whole system whereas local rates are determined at one step only with the concentrations of all potential effectors held constant. This distinction is important, because when we come to make a change in local rate and observe the change in flux the two changes will not necessarily be equal.

It is important to remember that it does not matter how the local rate change is brought about. It might be a hormone change, a change in gene expression or a change in apparent

K_m. Whatever it is, it does not matter at this stage of the argument. Thus the value of a control coefficient, C_v^J or C_v^S, will not depend on which particular parameter we choose to modulate.

Some workers (e.g. Crabtree & Newsholme, 1987; see also Atkinson in Chapter 36 of this book) have suggested that control coefficients are not appropriate, but they do in fact turn out to be central to the system view of metabolism. However, although the control coefficients are the central systemic properties they are not by themselves suitable candidates for measuring a metabolic regulation event as they do not reveal explicitly the role of the effectors in the regulatory response.

Response Coefficients: $R_p^J = \frac{\partial J}{\partial p} \cdot \frac{p}{J}$ and $R_p^S = \frac{\partial S}{\partial p} \cdot \frac{p}{S}$

Finally we come to the response coefficients. We use these to connect our pathway with the external world. They tell us how sensitive a system flux or a metabolite level is to changes in an external influence. We are obviously much closer to our goal of finding a coefficient that tells us something about regulation, for not only does it tell us what happens to the system but also what external influence induced the change. Here we also find an intimate relationship between elasticity coefficients and control coefficients. It is fairly easy to show (Kacser & Burns, 1973) that for some external effector I the product of the elasticity coefficient for I multiplied by the control coefficient of the step is the response coefficient. For example,

$$R_I^J = \varepsilon_I^v C_v^J$$

Even armed with the response coefficient, however, there still seems to be something missing and a feeling that there must be more to regulation. In particular, what of the role, if any, of *internal* effectors? There appears to be a whole network of internal effectors in metabolism. It is an assessment of how important such internal effectors are in contributing to a regulatory response that I now wish to make.

The Partitioned Regulatory Coefficient

Let us consider a particular example, in this case a simple linear chain with all steps reversible, thus:

$$X_0 \xrightarrow{1} S_1 \xrightarrow{2} S_2 \xrightarrow{3} S_3 \xrightarrow{4} S_4 \xrightarrow{5} X_5$$

X_0 and X_5 are clamped external metabolites with a net flux from left to right such that enzymes to the left and right of a step are termed *upstream* and *downstream* respectively. Let us consider a parameter change at the fifth step such that the local rate of this step is increased. The perturbation that results now ripples outwards, in this case upstream, causing changes in the metabolite levels all the way up to S_1 and in consequence a change ultimately

to the system flux. The degree to which the parameter change influences the flux and metabolite levels is given by the control coefficients. Assuming (to simplify the argument, but without losing generality in the conclusions) normal kinetics for each of the enzymes, i.e. that an increase in substrate concentration increases the local rate whereas an increase in product concentration lowers it, then, once the system settles down and reaches its new steady state we will find all metabolite concentrations to be lower, i.e.

$$C_{v_5}^{S} < 0$$

for all S. If we closely examine one step, say the second, we will observe three changes. Two of these will be lower values of S_1 and S_2 and the third will be a *higher* flux through the step. We can completely account for the higher flux through this step by the changes in S_1 and S_2. In fact, we can go further and examine how much either changes in S_1 and S_2 have contributed to the change in the flux. The decrease in S_1 will tend to lower the flux whereas the decrease in S_2 will tend to increase it. Obviously, therefore, the decrease in S_2 must have made a larger impact than the decrease in S_1 otherwise the flux would not have risen. To quantify this, let us consider the fractional change in net flux, $\delta J/J$ at step 2, and partition it into the two contributing (but opposite) changes to the local rate changes originating from S_1 and S_2, namely:

$$\delta J/J = \varepsilon_{S_1}^{v_2}\frac{\delta S_1}{S_1} + \varepsilon_{S_2}^{v_2}\frac{\delta S_2}{S_2}$$

The first term on the right is that contribution made by the change in S_1 and the second is the contribution made by the change in S_2. The changes in the substrate concentrations can be obtained from the concentration control coefficients, since

$$\frac{\delta S_1}{S_1} = C_{v_5}^{S_1}\frac{\delta v_5}{v_5} \quad \text{and} \quad \frac{\delta S_2}{S_2} = C_{v_5}^{S_2}\frac{\delta v_5}{v_5}$$

The partition can thus be rewritten as follows:

$$\delta J/J = \varepsilon_{S_1}^{v_2}C_{v_5}^{S_1}\frac{\delta v_5}{v_5} + \varepsilon_{S_2}^{v_2}C_{v_5}^{S_2}\frac{\delta v_5}{v_5}$$

We now have each contribution in terms of the original local rate change $\delta v_5/v_5$. Finally, by dividing throughout by $\delta J/J$ and using the definition of the flux control coefficient, we arrive at the following result:

$$1 = \varepsilon_{S_1}^{v_2}\left(C_{v_5}^{S_1}/C_{v_5}^{J_2}\right) + \varepsilon_{S_2}^{v_2}\left(C_{v_5}^{S_2}/C_{v_5}^{J_2}\right)$$

The two terms on the right-hand side, which are again dimensionless numbers, are examples of what we shall term the *partitioned regulatory coefficient*. This compound coefficient is almost identical to what was originally termed the *conditional elasticity* and first put forward by Holzhutter *et al.* (1985). I use the term *regulatory coefficient* to emphasize that it

is a system property and not simply an elasticity, and qualify it as *partitioned* because we have partitioned the different effects on the step The partitioned regulatory coefficients reveal quantitatively all the factors that contribute to the net response, namely the effects of the two changes in S_1 and S_2 on the local rates (the two elasticity coefficients for v_2), the sensitivity of S_1 and S_2 to the perturbation at step 5 (the two concentration control coefficients, and the effect on the flux (the flux control coefficient). I shall denote them symbolically by

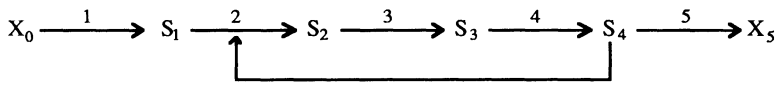
$$^{v_5}P^{J_2}_{S_1} = \varepsilon^{v_2}_{S_1}\left(C^{S_1}_{v_5}/C^{J_2}_{v_5}\right) \quad \text{and} \quad ^{v_5}P^{J_2}_{S_2} = \varepsilon^{v_2}_{S_2}\left(C^{S_2}_{v_5}/C^{J_2}_{v_5}\right)$$

Their values tell us, for a particular perturbation, what contribution the effectors around a particular step make in changing the flux through that step. Their relative magnitudes will depend on the site of perturbation. In general, perturbations upstream or downstream of a step will elicit different values for the regulatory coefficients. Intuitively this seems quite reasonable, since depending on where the pathway is perturbed we should get a different response.

In the example of the linear chain, it was noted that S_2 must make a larger contribution than S_1 in changing the flux at the step. This is easily verified as follows. Since we know that S_1 must make a negative contribution and S_2 a positive contribution, the partitioned regulatory coefficient for S_1 must be negative and that for S_2 must be positive, but since the sum of the coefficients must equal 1 the coefficient for S_2 must in that case be greater than 1. In fact, the coefficient for S_2 will always be greater (in absolute terms) than that for S_1 if we make the perturbation downstream from step 2. This example illustrates the importance of changes in product concentration in bringing about a regulatory response, an effect often ignored.

Feedback Systems

Discussion frequently centres on the role of negative feedback, as in the following scheme:



In such an instance we could assess the contribution the feedback makes to changing the flux at that step compared with the other effectors that might act on the flux, i.e. the substrate and product of the enzyme. The partitioning will now take the following form:

$$1 = {}^{v_5}P^{J_2}_{S_1} + {}^{v_5}P^{J_2}_{S_2} + {}^{v_5}P^{J_2}_{S_4}$$

in which

$$^{v_5}P^{J_2}_{S_4} = \varepsilon^{v_2}_{S_4}\left(C^{S_4}_{v_5}/C^{J_2}_{v_5}\right)$$

represents the feedback inhibition.

It is often assumed (without much evidence) that the feedback "regulates the flux". By

obtaining the magnitudes of the above coefficients, however, we can establish whether the third term is indeed the dominant one in this sum. Only then can we see the *relative* effects of the three effectors. It is by no means necessary that the negative-feedback term be dominant compared with the signal path along the main spine of the pathway. On the other hand, there may be a buffering effect along the pathway that could render the main pathway signal weaker compared with the direct feedback route. The degree to which this might be significant, however, will depend on the three elasticity coefficient coefficients for S_2, S_3 and S_4 on v_2. The summation of partitioned regulatory coefficients to unity implies that it is in principle possible to discover "missing effectors". The experimental determination of the values of the partitioned regulatory coefficients, based on certain structural assumptions about the pathway, will be found not to have a sum of unity if an important structural interaction is not included. Thus, in the last example, if the feedback partitioned regulatory coefficient is significantly different from zero its exclusion would cause the sum of the remaining two terms to deviate from unity.

It is, however, important to point out that this analysis is currently confined to the effects of small changes around a steady state. Analysis of large changes, i.e. induced large-scale transitions between two steady states, is invariably intractable mathematically. In general we must resort to numerical simulation to study such cases.

We may sum up by saying that the partitioning of the overall response into the contributions of each of the potential effectors gives a quantitative insight into the overall regulation.

Acknowledgements: Many thanks must be given to Dr H. Kacser for many hours of fruitful discussion and critical reading of this manuscript. The author is also grateful for financial support from the Wellcome Trust.

REFERENCES

Crabtree, B. & Newsholme, E. A. (1987) *Trends Biochem. Sci. 12,* 6-12
Holzhutter, H.-G., Jacobasch, G. & Bisdorff, A. (1985) *Eur. J. Biochem. 149,* 101-111
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104

Chapter 18

Determination of Control Coefficients by Shortening and Enzyme Titration of Metabolic Pathways

ENRIQUE MELÉNDEZ-HEVIA and NÉSTOR V. TORRES

THE GENERAL AIM of flux control analysis (Kacser & Burns, 1973; Heinrich & Rapoport, 1974) is to determine control distribution of a certain metabolic process among its different steps *in vivo,* for given physiological conditions. The relative importance of each step (enzyme or carrier) is quantified by its control coefficient, which expresses the fractional change produced in the selected flux, as a consequence of an infinitesimal fractional change in the enzyme activity. To obtain quantitative values of these control coefficients, experimental access to flux assay and modulation of enzyme activity are necessary. Normally these two requirements pose certain problems that are difficult to solve. *In vivo,* flux measurement is often not easy as all metabolic processes are interconnected. Usually, only in the processes that produce a metabolic end product, such as CO_2 or urea, or absorb an external substrate, such as glucose or amino acids, can flux *in vivo* be easily measured. The problem can become simplified in unicellular organisms, as their metabolic interactions are obviously simpler, but in any case nobody thinks that control analysis should be restricted to this type of organism. Even in the cases where the metabolic pathway implies a waste end-product or an external precursor, control analysis obliges us to define a precise metabolic pathway (which can involve branching) defined by one or several "external" substrates and products. The definition of these limits of the metabolic system is arbitrary (examples: glycolysis, pentose phosphate pathway, Krebs cycle etc). Therefore, the problem is not only the flux assay *in vivo,* but the delimitation of the metabolic pathway and the way of managing its function *in vivo* experimentally as well, a problem that is aggravated by the complexity of both organisms and metabolic pathways. The other problem is to obtain the modulation *in vivo* of all the enzymes (or at least some of them) implicated in the process. This can be done through genetic modulation or by means of specific inhibitors, but these two methods cannot always be used. However, if we transfer our problems to the field of experimentation *in vitro,* many of the problems mentioned can be solved. The method described here deals

Enrique Meléndez-Hevia and Néstor V. Torres ● Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de La Laguna, 38206 Tenerife, Canary Islands, Spain.

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

with the use of systems *in vitro* to study the distribution of control in metabolic pathways. We describe the general principles of application, which are based on familiar concepts and procedures of classical enzymology, although used to control analysis in whole systems. We shall give a general design and criticize its different features, in order to comment on the possibilities of its application to different types of systems. The method was first described by us (Torres *et al.*, 1985) and subsequently applied by our group to the control distribution in the first steps of rat-liver glycolysis under different physiological conditions (Mateo *et al.*, 1989; Torres *et al.*, 1988*ab*). Furthermore, on the basis of this method, a practical exercise has been proposed for initiating students in control analysis (Torres *et al.*, 1988*c*).

General Description of the Method

This method is based on obtaining a soluble cellular fraction in which activities of all enzymes of the metabolic pathway studied are in the same proportion as they occur in the whole system *in vivo*. In these conditions the reaction velocity the system *in vitro* through all the enzymes is less than that *in vivo*, by the same factor, but the concentrations of all intermediate pools will be the same as in the whole system *in vivo*. *In vitro* assays can include the whole pathway, with all the enzymes being coupled, or it can be shortened in several segments, each with a certain number of enzymes of the sequence. In every case flux can be measured by driving the end-product towards an appropriate substance by means of auxiliary enzymes, in the same way that enzyme activity assays are currently carried out. Then, the activity of each enzyme can be modulated by means of enzyme titration of the system in different series of experiments; thus, by assaying the flux, an empirical function that relates the flux with each enzyme activity of the system is obtained, and it allows determination of flux control coefficients. The values of these control coefficients are not the same as the whole system *in vivo;* they will be greater, since the system *in vitro* has fewer enzymes and in both cases the summation property must be satisfied; however, as will be shown below, a known relationship exists between them that will allow us to calculate them.

Methodology

Definition of the Metabolic Pathway. This is not a trivial step in the research. The metabolic pathway (we should say the "model" of metabolic pathway) is an abstraction to which we are habituated by textbooks. We must make sure that this model agrees as well as possible with physiological reality; this decision includes the organism, the cellular fraction where it occurs and a set of given physiological conditions. In our study, the design of the model is based on the choice of "external" substrates, products and effectors, and in the flux of transformation whose control we want to study. Although it is assumed that the fragment of metabolism that we have defined as the "metabolic pathway" has a physiological meaning in itself *in vivo*, its definition is in fact arbitrary; therefore, its relationship with other metabolic processes can generate certain problems that we shall discuss below.

Information about the System. The set of experimental protocols mentioned in this section are not really specific of the control analysis; therefore we will not take too long over their

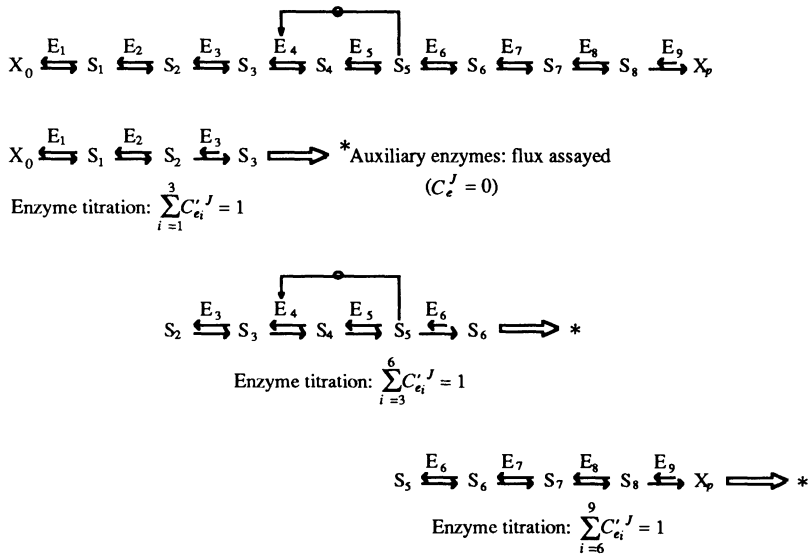
description. They are necessary to design the specific experimentation of control analysis, and to process results properly:

(*a*) *Assay of concentrations* of all the substrates, effectors and intermediate metabolites in the original situation *in vivo*. It must be demonstrated that dilution of the system from conditions *in vivo* to the conditions for working *in vitro* by a given dilution factor α produces a loss of activity in each enzyme by the same factor α.

(*b*) *Kinetic study of all the enzymes* of the system, in order to obtain their kinetic parameters (V_{max}, K_m, etc), and also the knowledge of possible feed-back (and feed-forward) effects of intermediate metabolites and external substrates, products, and effectors, at concentrations previously determined. Existence of enzyme-enzyme interactions in the pathway should also be known (see Kacser, Sauro and Acerenza in Chapter 20 of this book).

Shortening of the Pathway. This point includes two parts: the first is the design of the shortening and the second is the experimental implementation of it *in vitro*.

(*a*) *Design of the shortened segments of the pathway*. Every segment of the whole pathway that will be assayed *in vitro* is a subsystem defined by the specific external substrates, products and effectors of this segment (Scheme 1). In certain cases, in accordance with the features of the pathway and experimental possibilities, assay of the whole pathway without shortening could be possible. If shortening is necessary, two consecutive segments must always include at least one common enzyme, as shown in Scheme 1.



Scheme 1. Design of the shortening and enzyme-titration of a whole metabolic pathway. Each asterisk (*) has the meaning indicated for the first three-enzyme fragment, i.e. the flux for each fragment is measured after adding sufficient auxiliary enzymes for their flux control coefficients to be negligible.

Every shortened segment is isolated by buffering the external substrates and effectors at appropriate concentration and removing the end-product by means of auxiliary enzymes; these must have enough activity so that the concentration of end product be virtually zero, the last reaction of the segment being irreversible. Flux through the shortened pathway is assayed by driving the end product with the auxiliary enzymes up to a measurable substance (continuous recording of a substance such as NADH is very convenient). Since the end-product is removed in the *in vitro* systems, the shortening point must be chosen where no feed-back interactions occur or where their importance is negligible, and we must ensure, for the same reason, that all feedback and feedforward loops are included in the same shortened segment. All these considerations, as well as experimental possibilities for buffering the different substrates and effectors, and for measuring the fluxes will lead to the more convenient design of experimental procedure.

(*b*) *Experimental systems in vitro*. Assays are carried out with a soluble extract of the biological system, removing the cellular fractions that do not intervene in the metabolic pathway. Assay of flux in each segment is similar to classical assays in enzymology, for determination of enzyme activities in tissue extracts: incubation of the extract with substrates and effectors of the enzyme, and addition of auxiliary enzymes to the extract, for coupling the end-product with other reactions to give a measurable substance. This is a similar assay, but here the system includes several enzymes, whose coupled activity is assayed. Provided the steady state of the system is obtained, we have basal values of flux and activity of each enzyme; these basal values represent the point where control coefficients will be calculated.

Enzyme Titration. Having the system *in vitro* at steady state, it is now titrated with several quantities of each enzyme, around to the basal conditions, in different series of experiments; by assaying the flux and knowing the total activity of each enzyme in every experiment, we will obtain a curve that relates the flux through the system to each enzyme activity (basal plus added), the other enzymes being at their basal values. From these curves and basal values, control coefficients of the system *in vitro* can be obtained directly. Enzyme titration can be carried out with enzymes from any biological source (normally commercial enzymes will be used). The only condition for using them is to have a function that relates the two different enzyme activities (those of our system and the commercial ones), normalizing their kinetic parameters so that their activities can be additive. In the case of Michaelis-Menten kinetics and in conditions of low saturation, activity is given by V_{max}/K_m, since velocity is proportional to this. This expression is, however, not valid if enzymes have a significant degree of saturation. Then additional experimentation is needed to obtain a function that relates the two activities. Enzyme titration can also by done by removing a certain amount of each enzyme by means of antibodies.

Calculation of Flux Control Coefficients

Titration curves express an empirical relationship between enzyme activity and flux of the shortened pathway in the steady state. In a number of cases described in the literature

(Kacser & Burns, 1973; Torres *et al,* 1986; Salter *et al.,* 1986; Torres *et al,* 1988*ab*) these relationship have been shown to be hyperbolic. Kacser & Burns (1973) have described a kinetic model of a metabolic pathway that has an explicit solution and accounts for this hyperbolic relationship. The model is based in two hypotheses: all the enzymes have reversible Michaelis-Menten kinetics and all the enzymes are in conditions of low saturation. This model can be extended to the case where the first enzyme is significantly saturated (Torres, 1985). This hyperbolic relationship can be described as follows:

$$J = \frac{Q_1^i \cdot e_i}{Q_2^i + e_i} \tag{1}$$

where J is the flux, e_i the enzyme activity and Q_1^i and Q_2^i are parameters of the system that include all kinetic and thermodynamic information with the exception of e_i. Making the partial derivative of eqn. (1) with respect to e_i and multiplying by the scaling factor we obtain the following expression:

$$C_{e_i}^J = \frac{\partial J}{\partial e_i} \cdot \frac{e_i}{J} = \frac{Q_1^i - J_0}{Q_1^i} \tag{2}$$

where J_0 is the basal flux (without addition of titration enzymes). In our experiments we have calculated flux control coefficients by means of eqn. (2). Note that Q_1^i has a clear physical meaning: it is the maximum flux for the pathway, obtained when the activity of E_i tends to infinity, i. e. the flux through the system after addition of sufficient enzyme E_i so that it in no way limits the flux, at basal activities of the others. The value of Q_1^i is easily obtained from the experimental data by inverting eqn. (1), in a plot similar to the Lineweaver-Burk or other similar plots. These equations and calculations have also been used by Salter *et al.* (1986) to process hyperbolic results. It must be observed that the hyperbolic relationship between flux and enzyme activity is not a necessary condition to carry out the method of shortening-enzyme titration described here. In general, calculation of flux control coefficients from enzyme titration curves is based in the empirical function $J(e_i)$, and any function can be useful for this.

Real Values of the Flux Control Coefficients

The value of each control coefficient determined *in vitro* by this procedure is not the same as that of the whole system, but greater, since in every shortened segment fewer enzymes participate in the summation property. This is, in fact, an advantage of the method, since the increased magnitude of control coefficients makes them easier to measure. However, a relationship that allows their calculation exists among them: as dilution of the system has maintained the ratios of activity between all the enzymes, and every shortened pathway has been reproduced *in vitro* with the same concentrations of external substrates and effectors, the flux J^{vitro} of every system *in vitro* will be related to the flux J^{vivo} of the whole system

in vivo according to $J^{vivo} = \alpha J^{vitro}$ (where α is the dilution factor). Thus, it follows from the summation theorem for intermediate pools that in the diluted system all intermediate pools are at the same concentrations as those that occur *in vivo*. This means that the elasticity of each enzyme for any intermediate has the same value in the two systems; then, by applying the connectivity theorem of flux to both systems we have, for any two consecutive enzymes E_1 and E_2, connected by the intermediate S, that ${C_1}^{vivo}/{C_1}^{vitro} = {C_2}^{vivo}/{C_2}^{vitro}$, where C_i is any control coefficient of the whole system *in vivo* or of the shortened system *in vitro*, as indicated. Application of the same argument to other enzymes of the same segment gives the following relationship:

$$\frac{C_1^{vivo}}{C_1^{vitro}} = \frac{C_2^{vivo}}{C_2^{vitro}} = \frac{C_3^{vivo}}{C_3^{vitro}} = \cdots \tag{3}$$

and for the general case:

$$\frac{C_i^{vivo}}{C_j^{vivo}} = \frac{C_i^{vitro}}{C_j^{vitro}} \tag{4}$$

This relationship asserts that the ratio between any two flux control coefficients of the system *in vitro* is the same as that of its corresponding one *in vivo*. Enzymes E_i and E_j do not have to be consecutive in the pathway. A complete relationship can be obtained for all the enzymes of the system by overlapping the different shortened segments of the pathway (see Scheme 1). This method does not give, in principle, the absolute values of flux control coefficients, but a relationship among them all. This information is nevertheless important, as it gives the hierarchy in control distribution among the enzymes of the pathway. Absolute values of the coefficients can be obtained from direct knowledge of only one of them by whatever method. Nevertheless, in the next section we shall discuss some other approaches to this.

Metabolic Relationships of the Pathway with Others

As has been mentioned above, the definition of our metabolic pathway is not a trivial step in the research and we must emphasize this. For reproducing a metabolic pathway *in vitro* the system should include a number of simplified systems that simulate the other cellular processes mainly interacting with it. [An instance of such procedure has been given by Groen *et al.* (1982) in the study of the control of mitochondrial respiration: they included hexokinase in their experimental system to account for ATP depletion by the system; processes that consume ATP. In that work the control coefficient of hexokinase was also determined and it represents the influence of all these processes in controlling mitochondrial respiration]. Therefore, inclusion of reactions like this in our system has two advantages: our metabolic pathway, although isolated, is less artificial since it is not totally disconnected from other metabolic processes, and we can also know the influence of such processes in its control. In that case, if the system has been precisely designed, application of the summation theorem to the whole system give us another equation that determines eqns. (3) and then the absolute values of all control coefficients can be directly obtained.

Results Obtained by Applying this Method

Control of rat liver glycolysis has been analysed by our group by using the method proposed here. First experiments on total rat-liver glycolysis, from glucose to lactate and glycerol-phosphate *in vitro* (Meléndez-Hevia *et al.*, 1984) showed that addition of hexokinase and phosphofructokinase enhanced the flux of production of both lactate and glycerol phosphate. Then, in order to analyse this fact precisely, the method of shortening and enzyme titration was then designed and flux control distribution in the common pathway through the three first enzymes was then quantified (Torres *et al.*, 1986). A more complicated system of rat liver glycolysis, including the convergent branching from glycogen (which involves a shortened pathway of five enzymes) was also analysed (Torres *et al.*, 1988*a*), and the effect of different physiological conditions on the control of this pathway has been studied (Torres *et al.*, 1988*b*). In all these results many different aspects of the theory have been demonstrated, and furthermore, some new properties for particular pathway structures have been found that have allowed their theoretical exploration and the discovery of new theorems (see Torres *et al.*, 1988*a;* Meléndez-Hevia *et al.*, 1990). In all cases, the methodology here presented has proved to be a useful tool for the study of metabolic systems.

Concluding Remarks

It can be seen that the method here proposed uses current procedures that are in normal use by most laboratories. This could allow many groups to use it without special difficulties, since no complicated techniques are in principle necessary for its application. Other tools available now or in the future will give more possibilities to this method. It should be noted that the method here proposed is not a "close-formula", but the principles of a general methodology, which must be adapted in each case in accordance with the peculiar features of the pathway studied. In the application of this method some specific problems can arise (those concerning the abstraction of the defined metabolic pathway will not be negligible). In the present paper we have considered some of these problems and proposed solutions. We think that the imagination of every group will be able to solve these or other ones not considered by us.

Acknowledgements: This work was supported by a research grant from Conserjería de Educación del Gobierno de Canarias, Ref. No. 13/01. 06. 88. We are grateful to Dr. Joaquín Sicilia for helpful discussions.

REFERENCES

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. & Tager, J. M. (1982) *J. Biol. Chem. 257,* 2754-2757
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Mateo, F., Torres, N. V. & Meléndez-Hevia, E. (1989) *Cell. Molec. Biol. 35,* 33-37
Meléndez-Hevia, E., Siverio, J. M. & Pérez, J. A. (1984) *Int. J. Biochem 16,* 469-476
Meléndez-Hevia, E., Torres, N. V., Sicilia, J. & Kacser, H. (1990) *Biochem. J. 265,* 195-202
Salter, M., Knowles, R. & Pogson, C. I. (1986) *Biochem. J. 234,* 635-647

Torres, N. V. (1985) Doctoral Thesis, Universidad de La Laguna
Torres, N. V., Mateo, F., Meléndez-Hevia, E. & Kacser, H. (1986) *Biochem. J. 234,* 169-174
Torres, N. V., Mateo, F., Sicilia, J. & Meléndez-Hevia, E. (1988*a*) *Int. J. Biochem. 20,* 161-165
Torres, N. V., Mateo, F. & Meléndez-Hevia, E. (1988*b*) *FEBS Lett. 233,* 83-86
Torres, N. V., Meléndez-Hevia, E. & Riol-Cimas, J. M. (1988*c*) *Biochem. Educ. 16,* 100-102
Torres, N. V., Souto, R. & Meléndez-Hevia, E. (1989) *Biochem. J. 260,* 763-769

Chapter 19

Control-Pattern Analysis of Metabolic Systems

JAN-HENDRIK S. HOFMEYR

How do fluxes and metabolite concentrations, the variables of metabolic systems, respond to a change in some system parameter, such as an enzyme concentration or the affinity of an enzyme towards one of its effectors? Can such systemic behaviour be explained purely in terms of local enzymic properties? These fundamental questions about metabolic behaviour have been successfully addressed by metabolic control analysis (Kacser & Burns, 1973; Heinrich & Rapoport, 1974; also in numerous chapters of this book) and biochemical systems theory (Savageau, 1969*abc*, 1976; also in Chapters 4 and 5 of this book by Savageau and Voit respectively). In the language of metabolic control analysis the answer amounts to expressing control coefficients, which quantify global systemic behaviour, in terms of elasticity coefficients, which describe local enzymic behaviour. Similar coefficients are defined in biochemical systems theory. It is immaterial whether one derives these expressions from the summation and connectivity relationships of metabolic control analysis or the power law equations of biochemical systems theory. In metabolic control analysis, several methods of solution involving matrix algebra have been developed (Fell & Sauro, 1985; Sauro *et al.*, 1987; Small & Fell, 1989; Westerhoff & Kell, 1986) and they allow for the analysis of flux and concentration control in metabolic pathways containing linear, branched, looped and moiety-conserved structures. These methods are eminently suitable for numerical control analysis, but can be tedious for obtaining the algebraic solution.

Unfortunately, many biochemists still tend to shy away from the application of the concepts and methods of control analysis and biochemical systems theory in their research and teaching. In my experience this is mainly the result of unfamiliarity and unease with the mathematical techniques required in the application of these frameworks. This chapter describes a non-algebraic method, called "control-pattern analysis", that solves the problem of expressing control coefficients in terms of elasticity coefficients by tracing "control patterns" directly on the metabolic diagram according to a simple set of rules (Hofmeyr,

Jan-Hendrik S. Hofmeyr ● Department of Biochemistry, Faculty of Natural Sciences, University of Stellenbosch, Stellenbosch 7600, Republic of South Africa

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

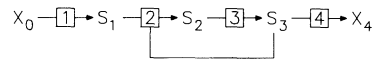


Figure 1. The example metabolic diagram of a four-enzyme linear pathway with a feedback loop. Enzymes are identified by boxes in unidirectional arrows which show the direction of the steady-state flux. All reactions are regarded as reversible in principle. X-metabolites are constant (external); S-metabolites are variable (internal). No S-metabolites should be shown more than once. Any regulatory loop (feedback and feedforward loop) is depicted by a line connecting the enzyme to the metabolite.

1989). Each control pattern of an enzyme can be translated into one of the terms in the expression for a specific control coefficient of that enzyme. Control-pattern analysis is analogous to the well-known method of King & Altman (1956) used in enzyme kinetics to derive steady-state enzymic rate equations. I shall show that control-pattern analysis not only generates the expressions for control coefficients, but that it also affords insight into the behaviour of metabolic systems in steady state.

The Method of Control Pattern Analysis

The summary of control-pattern analysis of linear pathways with regulatory loops described in this paper serves only to provide a basic idea of the procedure. A full description with examples can be found elsewhere (Hofmeyr, 1989), while control-pattern analysis of more complex systems with branches, loops and moiety-conserved cycles will follow.

A simple hypothetical metabolic pathway with one feedback loop (Fig. 1) serves as an example for introducing the procedure. The conventions used in drawing the metabolic diagram are explained in the legend to Fig. 1. In this linear pathway there is only one flux, which is numerically equal to the net rate of each of the four enzyme reactions. The set of special terms and diagrammatical entities that are associated with control-pattern analysis are depicted in Fig. 2.

The primary aim of control-pattern analysis is to obtain expressions for the flux-control coefficients in terms of elasticity coefficients. For the metabolic pathway in Fig. 1 the expressions for the flux-control coefficients are as follows:

$$C_1^J = \varepsilon_1^2\varepsilon_2^3\varepsilon_3^4/\Sigma \tag{1}$$

$$C_2^J = -\varepsilon_1^1\varepsilon_2^3\varepsilon_3^4/\Sigma \tag{2}$$

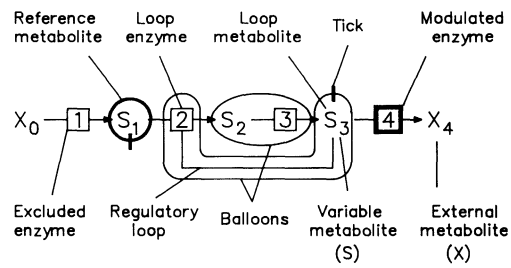


Figure 2. Entities in the diagrammatic representation of metabolic pathways and control patterns (an S_1-control pattern of E_4 serves as an example).

$$C_3^J = \varepsilon_1^1\varepsilon_2^2\varepsilon_3^4/\Sigma \tag{3}$$

$$C_4^J = \left(-\varepsilon_1^1\varepsilon_2^2\varepsilon_3^3 + \varepsilon_1^1\varepsilon_2^3\varepsilon_3^2\right)/\Sigma \tag{4}$$

whereas the expressions for, e.g., the S_1-control coefficients are as follows:

$$C_1^1 = \left(\varepsilon_2^3\varepsilon_3^4 - \varepsilon_2^2\varepsilon_3^4 + \varepsilon_2^2\varepsilon_3^3 - \varepsilon_2^3\varepsilon_3^2\right)/\Sigma \tag{5}$$

$$C_2^1 = -\varepsilon_2^3\varepsilon_3^4/\Sigma \tag{6}$$

$$C_3^1 = \varepsilon_2^2\varepsilon_3^4/\Sigma \tag{7}$$

$$C_4^1 = \left(-\varepsilon_2^2\varepsilon_3^3 + \varepsilon_2^3\varepsilon_3^2\right)/\Sigma \tag{8}$$

where Σ is defined as follows:

$$\Sigma = \varepsilon_1^2\varepsilon_2^3\varepsilon_3^4 - \varepsilon_1^1\varepsilon_2^3\varepsilon_3^4 + \varepsilon_1^1\varepsilon_2^2\varepsilon_3^4 - \varepsilon_1^1\varepsilon_2^2\varepsilon_3^3 + \varepsilon_1^1\varepsilon_2^3\varepsilon_3^2 \tag{9}$$

S_2-control and S_3-control coefficients are expressed similarly. Note that all control coefficients share the same denominator Σ, which is the sum of all numerator terms in the flux-control coefficients [eqns. (1-4)]. The flux or the metabolite concentration that is referred to by the superscript of a control coefficient will be called the *reference flux* or the *reference metabolite* respectively. The subscript refers to an enzyme which will be called the *modulated enzyme,* i.e. the enzyme the activity of which is considered to be altered by a small amount.

The general strategy of control-pattern analysis is first to determine the numerator of the flux-control coefficient (the *flux-control numerator*) of each enzyme in the pathway. Each term in the numerator with its associated sign corresponds to a diagrammatical "flux-control pattern". The numerators of flux-control coefficients are summed to form the denominator of the flux-control and concentration-control coefficients (the *control denominator*). The ratio of flux-control numerator to control denominator forms the expression for each flux-control coefficient. The numerators of the concentration-control coefficients (the *concentration-control numerators*) also correspond to a sum of "concentration-control patterns", determined by a procedure similar to that for flux-control numerators. The ratio of concentration-control numerator to the previously determined control-denominator forms the expression for each concentration-control coefficient.

The flux-control patterns for each enzyme (Fig. 3) are found by (i) defining the enzyme as the modulated enzyme by emphasizing the box around its identifier; (ii) drawing balloons around pairs of enzymes and S-metabolites that are connected with a diagram line, such that every S-metabolite occurs in one balloon; each balloon represents the elasticity coefficient of the enzyme for the metabolite; there are three types of balloons: substrate balloons contain an enzyme and its substrate, product balloons an enzyme and its product, and loop balloons an enzyme and its internal effector [the rules for drawing balloons follow from the properties of the control-matrix equation (Hofmeyr, 1989)]; (iii) tracing a *flux-control path* from the

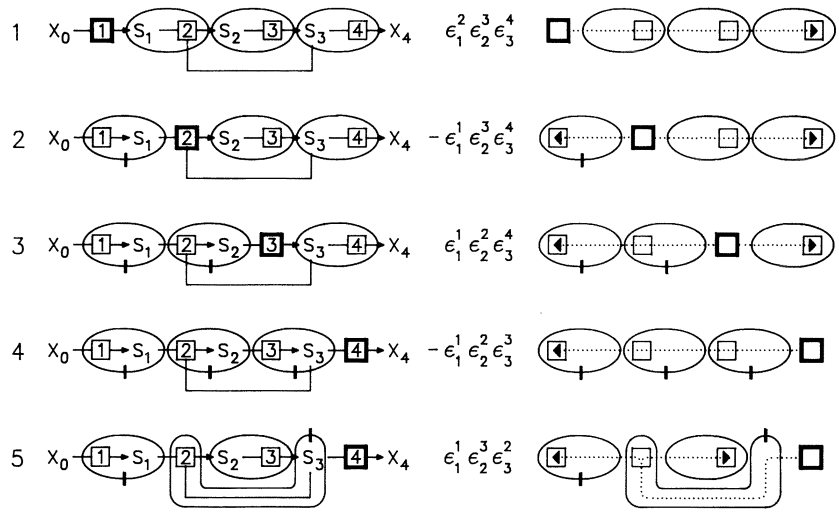


Figure 3. Flux-control patterns of all the enzymes in the example diagram. The flux-control path for each control pattern is shown on the right as a dotted arrow. This path runs on the diagram lines through consecutive balloons and shows one way in which the effects of an enzyme modulation can be transmitted through the pathway. To trace a flux-control path, start at the modulated enzyme and move into the end of an adjacent balloon that is connected directly to the modulated enzyme by a diagram line. Follow this balloon to its other end and move into a next directly connected balloon, following it to its end. Repeat this until the path comes to a dead end either at an X-metabolite or by having looped back onto itself. The path may fork at an enzyme, but it must not cross or retrace itself. If the path ends before all balloons have been traversed, start again at the modulated enzyme (or at an enzyme where a fork in the path is possible) and proceed in the opposite direction until the path stops.

modulated enzyme through consecutive directly adjacent balloons (see Fig. 3); (iv) using the flux-control path to determine the sign that precedes the product of elasticity coefficients that represents the flux-control pattern. This step comprises ticking all product balloons and those loop balloons in which the loop metabolite lies upstream from the enzyme that immediately precedes it in the flux-control path. An uneven number of ticks gives a – sign, an even number a + sign.

The balloons in concentration-control patterns are drawn in the same way as for flux-control patterns (Fig. 4 shows all the S_1-control patterns), except that the metabolite referred to by the concentration-control coefficient (the reference metabolite) is identified by drawing a circle around it. The reference metabolite must not appear in a balloon; therefore, one enzyme will necessarily fall outside a balloon — this enzyme is called the *excluded enzyme*. Each concentration-control pattern is composed of part of a flux-control pattern of the modulated enzyme and part of a flux-control pattern of the excluded enzyme. These partial patterns connect to opposite sides of the reference metabolite, and, together with their associated partial flux-control paths and (ticked or unticked) balloons, can be mapped directly onto each other to form the concentration-control pattern. The control path from modulated enzyme to reference metabolite is called the *concentration-control path*. In determining the pattern sign one extra ticking rule is used: the reference metabolite must be

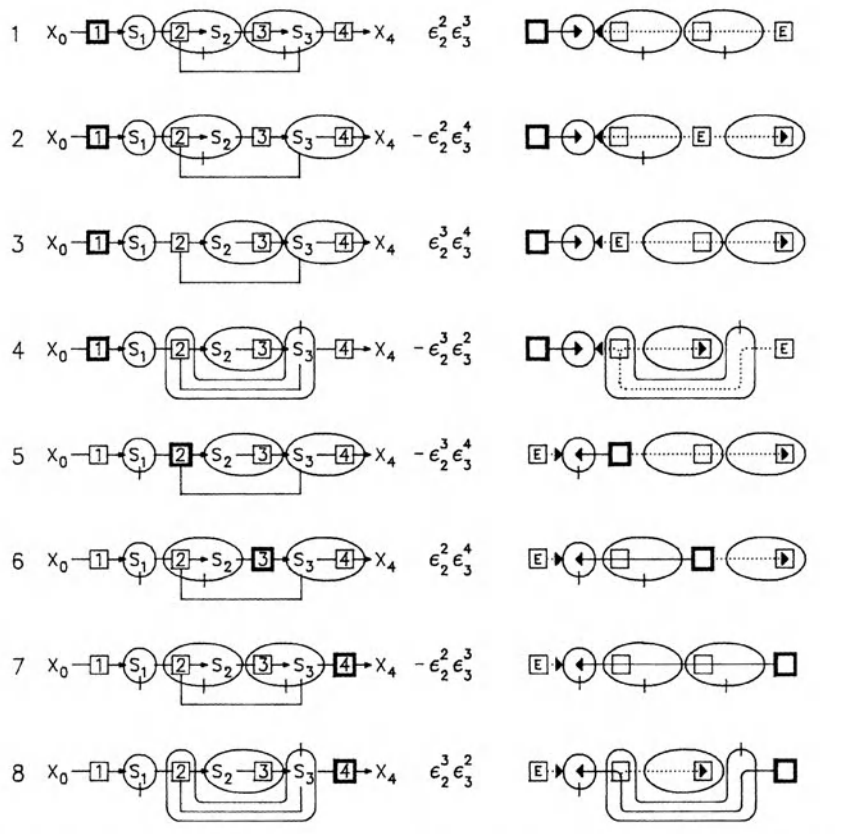


Figure 4. S_1-control patterns of all the enzymes in the example diagram. The concentration-control path for each control pattern is shown on the right as a solid arrow. Other control paths are shown as dotted arrows. The boxed E indicates the excluded enzyme.

ticked if it lies upstream from the enzyme that immediately precedes it in the concentration-control path. Fig. 5 explains the above procedure diagrammatically.

A Physical Interpretation of Control Patterns

Control-pattern analysis has been designed in such a way that it not only generates the expressions for control coefficients in terms of elasticity coefficients, but also provides the means by which the events that follow a modulation of enzyme activity can be described in physical terms.

Consider the flux-control pattern $-\epsilon_1^1\epsilon_2^3\epsilon_3^4$ for E_2 (pattern 2 in Fig. 3). In the absence of unusual kinetic features, elasticities towards substrates are positive, while those towards products are negative, i.e. an increase in substrate concentration increases the reaction rate, while an increase in product concentration decreases the rate. Therefore this flux-control

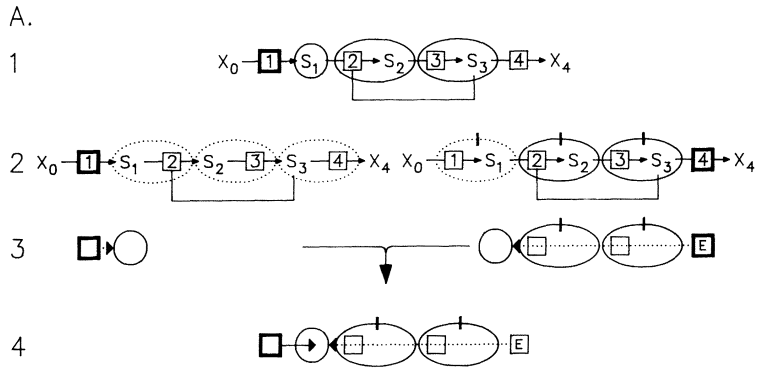
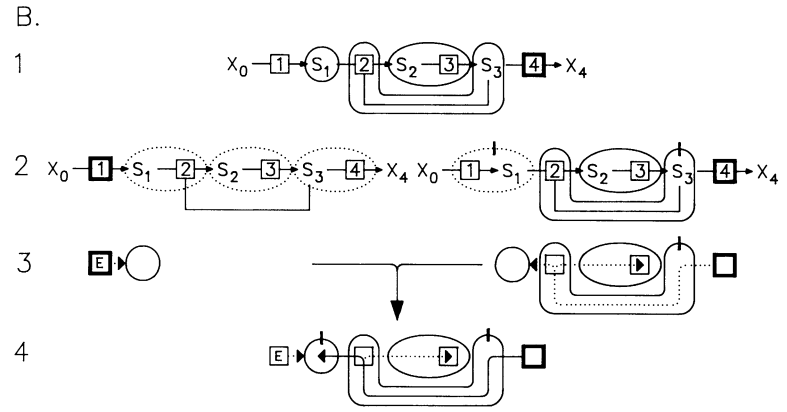


Figure 5. Control paths in S_1-control patterns 1 (A) and 8 (B, opposite) in Fig. 4. Each part of A and B shows: (1) the concentration-control pattern; (2) the two flux-control patterns which correspond partially to the concentration-control pattern (the dotted balloons do not fit); (3) the partial flux-control paths in the corresponding parts of the patterns (dotted arrows); (4) the combination of the partial flux-control paths from (3) to form the control paths in the concentration-control pattern. The path from the modulated enzyme to the reference metabolite (solid arrow) is extended into the circle to indicate the concentration-control path; the dotted arrows form other control paths from the modulated enzyme or the excluded enzyme (boxed E).

pattern must be positive under normal conditions (the product of two positive substrate elasticities, ε_2^3 and ε_3^4, and one negative product elasticity, ε_1^1, is preceded by a minus sign). Note that the sign part of the pattern is taken into account when deciding whether a flux-control pattern is denoted positive or negative.

A positive flux-control pattern for E_2 implies that the flux, J, is increased via that pattern when the activity of E_2 is increased; this means equal fractional increases in the net steady-state rates of all enzymes in this pathway. By analysing the flux-control pattern for E_2 as a "chain of local effects" that flows along the flux-control path after modulation of this enzyme, one can show that these changes must occur under normal conditions. An increase in the activity of E_2 will decrease S_1, which, in turn, will increase the local rate v_1. S_2 (the product of E_2) will increase, with a subsequent increase in the local rate v_3. The change in v_3 increases v_4 through an increase in S_3. Qualitatively, this change of local effects can be symbolized as $\uparrow v_2 \downarrow S_1 \uparrow v_1$ and $\uparrow v_2 \uparrow S_2 \uparrow v_3 \uparrow S_3 \uparrow v_4$, where up-arrows show an increase in a rate or concentration, and down-arrows a decrease. Since all rates increase, the flux-control pattern for E_2 is positive. In the same way, the flux-control patterns for E_1 and E_3 are positive; the chain of local effects for E_1 is $\uparrow v_1 \uparrow S_1 \uparrow v_2 \uparrow S_2 \uparrow v_3 \uparrow S_3 \uparrow v_4$, and those for E_3 $\uparrow v_3 \uparrow S_3 \uparrow v_4$ and $\uparrow v_3 \downarrow S_2 \uparrow v_2 \downarrow S_1 \uparrow v_1$. The chain of local effects along a flux-control path therefore shows how the effects of an enzyme modulation are propagated through the pathway and is used as an aid to understanding why a control pattern is positive or negative.

Now consider the flux-control patterns of E_4, the enzyme downstream from the loop (patterns 4 and 5 in Fig. 3). There are two ways of pairing enzymes and metabolites in balloons. S_3 can be linked to either E_3 or, via the feedback loop, to E_2; both lead to valid flux-control patterns in which all metabolites are paired with enzymes. Under normal conditions $-\varepsilon_1^1 \varepsilon_2^2 \varepsilon_3^3$ is positive (the chain of local effects is $\uparrow v_4 \downarrow S_3 \uparrow v_3 \downarrow S_2 \uparrow v_2 \downarrow S_1 \uparrow v_1$). When the regulatory loop is inhibitory ($\varepsilon_3^2 < 0$), the pattern $\varepsilon_1^1 \varepsilon_2^3 \varepsilon_3^2$ is positive: $\uparrow v_4$ causes a



decrease in S_3, thereby relieving the inhibition of E_2 by S_3 and causing an increase in v_2 (the chain of local effects is $\uparrow v_4 \downarrow S_3 \uparrow v_2 \downarrow S_1 \uparrow v_1$ and $\uparrow v_2 \uparrow S_2 \uparrow v_3$). The net positive effect on the metabolic flux of a modulation in E_4 is clearly the result of a superimposition of two separate positive flux-control patterns.

If the feedback loop is activatory ($\varepsilon_2^3 > 0$), flux-control pattern $\varepsilon_1^1 \varepsilon_2^3 \varepsilon_3^2$ for E_4 is negative; the chain of local effects is $\uparrow v_4 \downarrow S_3 \downarrow v_2 \uparrow S_1 \downarrow v_1$ and $\downarrow v_2 \downarrow S_2 \downarrow v_3$. It is clear that positive feedback could lead to a negative C_4^J. The final effect of an increase in the activity of E_4 would depend on the relative magnitudes of the positive flux-control pattern ($-\varepsilon_1^1 \varepsilon_2^2 \varepsilon_3^3$) and the negative flux-control pattern ($\varepsilon_1^1 \varepsilon_2^3 \varepsilon_3^2$). However, while the numerator may become negative if the activatory loop pattern dominates the chain pattern, the denominator must remain positive if the steady state is to remain stable. This is a general truism: if the denominator of any set of control coefficients (as determined by control-pattern analysis) becomes negative, those control coefficients describe an unstable steady state; if the denominator is zero, the steady state does not exist (Hofmeyr, 1986).

Just as flux-control patterns have a positive or a negative effect on a flux, depending on the values of the elasticity coefficients, concentration-control patterns also act to either increase or decrease the concentration of the reference metabolite. Consider, for example, the eight S_1-control patterns in Fig. 4. If substrate balloons represent positive elasticities, product balloons negative elasticities and the feedback loop is inhibitory ($\varepsilon_3^2 < 0$), the four patterns of E_1 are all positive. As required by the summation property of concentration-control coefficients, which states that the set of control coefficients for any one metabolite must sum to zero (Westerhoff & Chen, 1984), the remaining four patterns, one each for E_2 and E_3 and two for E_4, are the negative counterparts of the four S_1-control patterns of E_1. A chain of local effects can be followed along the concentration-control path just as in flux-control paths. For instance, in the negative S_1-control patterns of E_4 (7 and 8 in Fig. 4) the chains are $\uparrow v_4 \downarrow S_3 \uparrow v_3 \downarrow S_2 \uparrow v_2 \downarrow S_1$ and $\uparrow v_4 \downarrow S_3 \uparrow v_2 \downarrow S_1$ respectively.

Although all the S_1-control patterns of E_1 (the enzyme that lies upstream from S_1) are positive, and all the S_1-control patterns of the other enzymes (which lie downstream from S_1) are negative, this is not always so. As as exercise you could write the three S_2-control patterns of E_4 and analyse them. The $-\varepsilon_1^1 \varepsilon_3^3$ and $\varepsilon_1^2 \varepsilon_3^3$ patterns are negative, while, if the

feedback loop is inhibitory, the $\varepsilon_1^1\varepsilon_3^2$ pattern is positive. Thus, whether C_4^2 is positive or negative depends on the relative magnitude of the three patterns.

The concept of a chain of local effects provides a way of explaining the logical basis of the procedure for determining the sign of any control pattern. The ticking rules ensure that, as the chain of local effects is followed along any control path, the elasticities are given such values that all the rates increase. This means that all flux-control patterns are forced to be positive, while the positive or negative nature of concentration-control patterns depends only on the position of this metabolite relative to the enzyme that precedes it in the concentration-control path. If there are balloons in a concentration-control pattern that can only be accommodated in control paths other than the concentration-control path, the ticking rules ensure that all of these balloons are given such values that they cannot change the control-pattern sign. Of course, once the correct sign for a control pattern has been determined, any elasticity value can be considered, so that the expressions for control coefficients can be used to analyse the effects of, for example, substrate inhibition or feedback activation.

Control Patterns in more Complex Pathways

Control-pattern analysis also treats complex metabolic pathways that contain branches, loops and moiety-conserved cycles. The basic procedure remains the same, but a few extra rules must be applied (Hofmeyr, 1986).

In branched pathways, where there is more than one flux, a reference flux must be chosen. In a flux-control pattern, every enzyme in the reference flux must either occur in a balloon or it must be the modulated enzyme. Any other flux may contain only one excluded enzyme. The flux-control path connects the modulated enzyme to the reference flux. Furthermore, each pattern is weighted by that flux or product of fluxes in which there is no excluded enzyme; since the reference flux weights all flux-control patterns it cancels. Fig. 6 shows the flux-control patterns of two enzymes in a branched pathway: E_1, which lies in the reference flux J_a, has four patterns, while E_3 and E_5, which lie in J_b and J_c respectively, have two patterns each. There is one extra ticking rule for determining the sign of a pattern: if, in any control path, fluxes converge at or diverge from a branchpoint metabolite, the balloon in which it occurs is ticked, irrespective of whether it has been ticked before. For example, if the direction of J_b in J_a-control patterns 1 and 2 of E_1 is reversed, both ε_2^3 and ε_3^4 become product balloons and are ticked; ε_2^3 is ticked again since J_a and J_b converge at S_2.

Metabolic loops are formed when branches reconverge. All the rules for branched systems apply, but the flux-control path(s) depend on whether loop fluxes or non-loop fluxes are chosen as reference (Hofmeyr, 1986). This aspect will not be illustrated here.

Moiety-conserved cycles (Hofmeyr *et al.*, 1986) are formed when a set of reactions interconvert different forms of a chemical moiety, while the sum of the concentrations of these forms remains constant; a common example is the NAD^+/NADH cycle. Control patterns of enzymes in pathways containing such cycles are found according to the rules for linear systems. There will always be one cycle metabolite that cannot be accommodated in a balloon. The concentration of this metabolite, expressed as a fraction of the sum of the

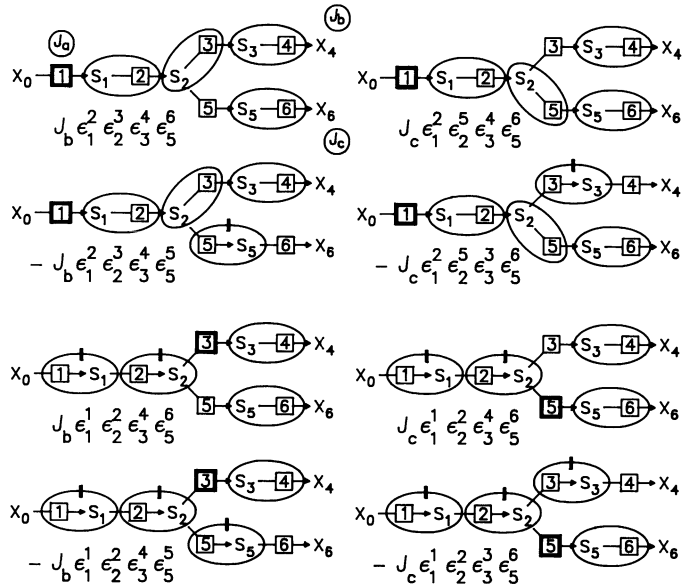


Figure 6. Flux-control patterns in a branched pathway. There are three fluxes (J_a, J_b and J_c) of which J_a has been chosen as the reference flux. The J_a-control patterns of an enzyme that lies in the reference flux (E_1) and two enzymes that lie in other fluxes (E_3 and E_5) are shown. Note that each pattern is weighted by the flux (other than the reference flux) in which there are no excluded enzymes.

concentrations of cycle metabolites, weights the product of elasticities that represents the control-pattern. Fig. 7 shows the E_1 and E_3 flux-control patterns in a pathway that contains a 3-member moiety-conserved cycle.

Conclusion

The advantages of control-pattern analysis are many: it is as general as any of the algebraic methods for obtaining the expressions for control coefficients, but no algebra is required in its application; the type of metabolic diagram on which control patterns are traced is familiar to all biochemists; furthermore, each control pattern can be understood in physical terms as a chain of local effects following the modulation of an enzyme activity, the final response being a superimposition of the individual chains of effect. In such a way the complex set of events that follow an activity modulation can be dissected into individual positive or negative patterns in which the systemic role of every link or effect between metabolites and enzymes is clear

At present, the technique of control-pattern analysis can treat pathways subject to the following restrictions: (i) no intermediary metabolite may be repeated on the metabolic diagram; (ii) only combinations of four metabolic structures are allowed, namely linear chains, branched chains, metabolic loops (futile loops and parallel loops) and moiety-conserved cycles; (iii) enzyme concentrations are regarded as constant; variable metabolites

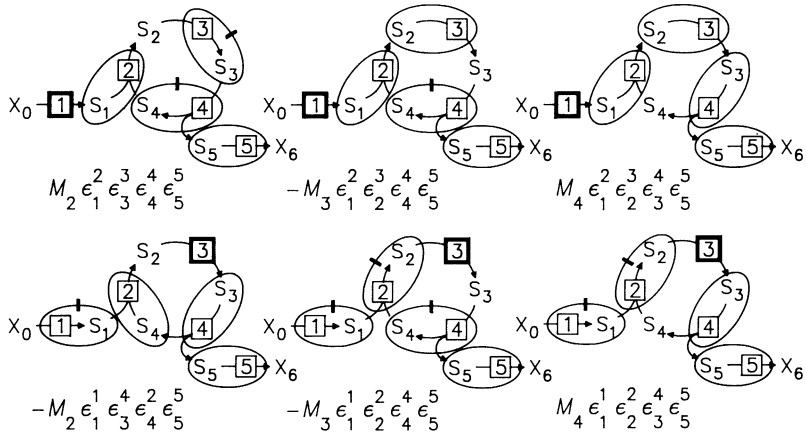


Figure 7. Flux-control patterns in a pathway that contains a moiety-conserved cycle. The top three patterns are those of a non-cycle enzyme (E_1), while the bottom three are those of a cycle enzyme (E_3). Note that each pattern is weighted by a mole fraction, e.g. M_2, where $M_2 = S_2/(S_2+S_3+S_4)$. The mole-fraction weight is always that of the cycle metabolite that does not occur inside a balloon.

exhibit free pool behaviour. Enzyme-enzyme interactions, subunit association-dissociation, channelling of intermediary metabolites, enzyme cascades and repression-induction fall outside the scope of the method at present. However, an enzyme cascade or a channelled part of the pathway could be lumped into a single "conversion unit" with elasticity coefficients towards variable metabolites on either side of the unit, after which it can be treated in the normal way. Within these restrictions control-pattern analysis is a powerful conceptual tool for understanding the relationship between local and systemic properties of most types of metabolic pathway, without the need for possibly unfamiliar algebra.

REFERENCES

Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95
Hofmeyr, J.-H. S. (1986) Ph.D. Thesis, University of Stellenbosch
Hofmeyr, J.-H. S. (1989) *Eur. J. Biochem. 186,* 343-354
Hofmeyr, J.-H. S., Kacser, H. & Van der Merwe, K. J. (1986) *Eur. J. Biochem. 155,* 631-641
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
King, E. L. & Altman, C. (1956) *J. Phys. Chem. 60,* 1375-1378
Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221
Savageau, M. A. (1969*a*) *J. Theor. Biol. 25,* 365-369
Savageau, M. A. (1969*b*) *J. Theor. Biol. 25,* 370-379
Savageau, M. A. (1969*c*) *J. Theor. Biol. 26,* 215-226
Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts
Small, J. R & Fell, D. A. (1989) *J. Theor. Biol. 136,* 181-197
Westerhoff, H. V., & Chen, Y. (1984) *Eur. J. Biochem. 142,* 425-430
Westerhoff, H. V., & Kell, D. B. (1986) *Biotechnol. Bioeng. 30,* 101-107

Part V

CHANNELLING OF INTERMEDIATES AND THE TIME DOMAIN

Chapter 20

Control Analysis of Systems with Enzyme-Enzyme Interactions

HENRIK KACSER, HERBERT M. SAURO and LUIS ACERENZA

THE CLASSICAL approach to control analysis has made two fundamental assumptions: the first is that all enzymes are independently acting catalysts, and the second is that the reaction rate of an isolated enzyme is first-order with respect to enzyme concentration. The first assumption, that of *independence,* implies that no direct interactions between enzymes affect their catalytic activities (other than that which occurs indirectly through the common substrate or effector pools). This assumption would be invalid, for example, if dynamic enzyme-complex formation affected the kinetic parameters of the constituent enzymes. The second assumption, that of *additivity,* based on classical enzymology, would not apply if, for example, an enzyme oligomer-monomer equilibrium existed for a single reaction step, or if the enzyme were partitioned between free and membrane-bound forms, the membrane being present in fixed amount, with different activities of the two forms.

The control coefficients can be defined, quite generally, as system responses with respect to changes in the "local rate" v of a reaction step (without specifying the parameters responsible for the change). The coefficients are defined as follows:

$$C_{v_i}^{J_j} = \frac{v_i}{J_j}\left(\frac{\partial J_j}{\partial v_i}\right)_{v_j,\, v_k \ldots}$$

$$C_{v_i}^{S_j} = \frac{v_i}{S_j}\left(\frac{\partial S_j}{\partial v_i}\right)_{v_j,\, v_k \ldots} \qquad (1)$$

where J_j is any one flux in the system and S_j any one metabolite. The summation and connectivity theorems (Kacser & Burns, 1973, 1979; Heinrich & Rapoport, 1974; Kacser, 1983; Westerhoff & Chen, 1984) then follow directly:

$$\text{Summation theorems: } \sum_{i=1}^{n} C_{v_i}^{J_j} = 1; \quad \sum_{i=1}^{n} C_{v_i}^{S_j} = 0 \qquad (2a)$$

Henrik Kacser, Herbert M. Sauro and Luis Acerenza ● Department of Genetics, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

$$\text{Connectivity theorems: } \sum_{i=1}^{m} C_{v_i}^{J_j} \varepsilon_k^i = 0; \quad \sum_{i=1}^{m} C_{v_i}^{S_j} \varepsilon_k^i = -\delta_{jk} \tag{2b}$$

where $\delta_{jk} = 1$ for $j = k$ and 0 for $j \neq k$. The summation over all n enzymes in the system constitutes a constraint on the values of individual coefficients. The connectivity theorems relate the coefficients of the m enzymes that interact with a given metabolite S_k (which may be a substrate, product or other effector of the enzymes), to the metabolite elasticity coefficients ε_k^i. These are defined for *any* rate, "isolated" from the rest of the system, as follows:

$$\varepsilon_k^i = \frac{S_k}{v_i}\left(\frac{\partial v_i}{\partial S_k}\right)_{S_i, S_j \ldots} \tag{3}$$

i.e. the scaled or normalized partial derivative of a "local" rate equation with respect to any one of the concentrations of the molecules that participate in the reaction. The rate equation can be of any form and is not restricted to simple mechanisms. Even if unknown, the metabolite elasticities can be determined empirically (Kacser & Burns, 1979). There are as many elasticity coefficients for any one rate as there are participating molecules in the rate equation. All the elasticity coefficients of all the rates in the system, when defined for the steady state, represent the potential response of the isolated rates to perturbations transmitted via metabolite movements. The matrix of all the elasticities jointly generates the "system" responses, i.e. the control coefficients $C_{v_i}^{J_j}$ and $C_{v_i}^{S_j}$ (Kacser & Burns, 1973; Kacser, 1983, Fell & Sauro, 1985), and each control coefficient can be expressed in terms of different functions of the metabolite elasticities (see, e.g., Kacser & Porteous, 1987, or Chapter 3 by Porteous in this book). Subsequent to the establishment of the above theorems (Kacser & Burns, 1973; Heinrich & Rapoport, 1974), it has become quite clear that one can start with apparently quite different descriptions (although the same basic kinetic model) and use different conceptual routes. All such approaches (Savageau, 1976; Reder, 1988; Giersch, 1988*ab*, Cascante *et al.*, 1989; also numerous chapters in this book) lead, however, to exactly the same conclusions as the theorems of eqns. (2)[1]. It is these agreed properties of biochemical systems that form the starting point of further developments in our work.

Parameter Elasticity Coefficients

The theorems as formulated in eqns. (2) are valid irrespective of any interactions between enzymes that may obtain, since the coefficients are defined in eqns. (1) without specifying how the changes may be brought about. To effect a change in the local rate, a parameter directly involved in the rate equation for the step must be modulated. The relationship between rate and parameter is given by

---

[1]The ultimate priority claim, however, must go to Leonhard Euler (1707-1783), who established the properties of homogeneous functions of degree 1, the equivalent of the summation theorems (see Giersch, 1988 *ab*; also Chapter 30 by Giersch, Lämmel and Steffen in this book).

$$\frac{\delta v_i}{v_i} = \pi_j^i \frac{\delta p_j}{p_j} \tag{4}$$

where it is useful to designate the elasticity coefficient with respect to any parameter p in the rate equation (K_m, E, K_i etc.) by the symbol π:

$$\pi_j^i = \frac{p_j}{v_i}\left(\frac{\partial v_i}{\partial p_j}\right)_{p_k,\, p_l} \tag{5}$$

and hence is defined (and measurable), again, for the "isolated" condition. When the parameter considered is the enzyme concentration E_j,

$$\pi_j^i = \frac{E_j}{v_i}\left(\frac{\partial v_i}{\partial E_j}\right) \tag{5a}$$

Additivity: Effects at a Single Step, $j = i$

If we consider the enzyme whose concentration E_i occurs in its "own" rate equation, the π elasticity is as follows

$$\pi_i^i = \frac{E_i}{v_i}\left(\frac{\partial v_i}{\partial E_i}\right) \tag{5b}$$

Clearly, if the rate is proportional to enzyme concentration,

$$\pi_i^i = 1 \qquad \text{(Additivity)} \tag{6}$$

For non-proportionality,

$$\pi_i^i \neq 1 \qquad \text{(Non-additivity)} \tag{7}$$

Independence: Interactions between Steps, $j \neq i$

For all enzymes acting independently, no effects of changes in any enzyme concentrations E_j affect any rate v_i and therefore

$$\pi_j^i = 0 \qquad \text{(Independence)} \tag{8}$$

If any kinetically significant interactions do take place, then for these

$$\pi_j^i \neq 0 \qquad \text{(Non-independence)} \tag{9}$$

Effects on the Summation and Connectivity Theorems

With additivity and independence assumptions, each control coefficient C_v can be replaced by C_E, with E replacing v:

$$C_{E_i}^{J_j} = \frac{E_i}{J_J}\left(\frac{\partial J_j}{\partial E_i}\right)$$
$$C_{E_i}^{S_j} = \frac{E_i}{S_J}\left(\frac{\partial S_j}{\partial E_i}\right) \qquad (10)$$

and the theorems are exactly the same as for coefficients C_v, i.e eqns. (2), with E replacing v everywhere.

These are the formulations of the theorems most frequently encountered in both theoretical and experimental publications on control analysis. Additivity and independence are the normal assumptions based on "solution chemistry" and metabolic map "reconstruction". *In vivo,* however, these assumptions must not be taken for granted, and there is increasing evidence that, not infrequently, the assumptions may be violated. We must therefore enquire how non-additivity and non-independence, defined above, will affect the theorems and modify the control distribution.

The Π *Matrix*

Taking non-additivity first, as the simpler problem, it can be shown (Kacser *et al.,* 1990) that if, at any step, we have non-proportionality of rate with enzyme concentration, the C_E and C_v coefficients are unequal, but we have the following relationship

$$C_{E_i}/\pi_i^j = C_{v_i} \qquad (11)$$

for flux or concentration control coefficients.

As a result, if modulation of enzyme concentrations is carried out, the summation theorems, which apply to C_v coefficients, must be modified from

$$\sum_{i=1}^{n} C_{v_i}^{J_j} = 1; \quad \sum_{i=1}^{n} C_{v_i}^{S_j} = 0 \qquad (12)$$

[cf. eqns. (2)] to

$$\sum_{i=1}^{n} C_{E_i}^{J_j} = 1 + \mathrm{Dev}_1; \quad \sum_{i=1}^{n} C_{E_i}^{S_j} = 0 + \mathrm{Dev}_2 \qquad (13)$$

where Dev_1 and Dev_2 represent the deviations from the behaviour with additivity. A knowledge of the π values involved, however, would yield:

$$\sum_{i=1}^{n} C_{E_i}^{J_j}/\pi_i^j = 1 \qquad (14)$$

allowing for any or all of the steps to have non-unit π_i^j values. Similarly,

$$\sum_{i=1}^{n} C_{E_i}^{S_j}/\pi_i^j = 0 \tag{15}$$

As an example one can consider the case where the enzyme consists of various homo-oligomers. For instance, a monomer-dimer system will be governed by an equilibrium constant K = [dimer]/[monomer]^2, so that, if the activity (per unit site) on the dimer is different from that of the monomer, enzyme modulation would shift the equilibrium between the two forms, and non-additivity ($\pi_i^j \neq 1$) would result. A number of such cases are discussed in Kacser *et al.* (1990).

Turning now to the association of different enzymes to form complexes (Srere, 1987; see also Chapters 21-22 by Keleti and Ovádi respectively in this book), it will be possible that such association affects the kinetic properties of the constituent parts. This implies that the particular $\pi_j^j \neq 0$ and that the enzymes act non-independently in their catalytic functions. Again, modulation of one enzyme concentration would affect not only its own activity but also that of its associated enzyme in that the concentration of the complex (and hence the net activity) would change. The formulations for such cases are more complex than for the non-additive case, but both types of enzyme-enzyme interactions can be quantified by a Π matrix that pre-multiplies the elasticity matrix (Fell & Sauro, 1985; Sauro *et al.*, 1987; Sauro & Kacser, 1990; Sauro & Small, unpublished work). This Π matrix contains all the possible non-additive and non-independent π terms. The diagonal entries comprise the non-additive π_i^j terms and the non-diagonal entries the non-independent π_j^j terms.

The relationships between $\mathbf{C_v}$ and $\mathbf{C_E}$ are given in matrix notation by

$$\begin{aligned} \mathbf{C_E^J} &= \Pi^T \mathbf{C_v^J} \\ \mathbf{C_E^S} &= \Pi^T \mathbf{C_v^S} \end{aligned} \tag{16}$$

As the theorems of eqns. (2) apply to coefficients C_v, a new, more general set is given for coefficients C_E, in which [1] represents a unit vector:

$$\begin{aligned} \text{Flux summation theorem: } & \left[\mathbf{C_E^J}\right]^T \Pi^{-1}[1] = 1 \\ \text{Concentration summation theorem: } & \left[\mathbf{C_E^S}\right]^T \Pi^{-1}[1] = 0 \end{aligned} \tag{17}$$

Similar transformations apply to the connectivity theorems.

Thus if there are interactions in the system we can modify the experimentally found C_E by their respective π elasticities to give C_v values to which the various theorems apply. The π values themselves are experimentally accessible. Thus, the non-additive π of eqn. (5*b*) can be written as follows:

$$\pi_i^j = \frac{\partial \ln v_i}{\partial \ln E_i}$$

so the slope of a plot of log v against log E will give a non-unit value if there is non-proportionality. The slope will vary with the absolute value of E_i and the value relevant to the *in vivo* condition must be chosen.

Similarly, for non-independence, "isolated" enzyme pairs will have to be investigated for the deviation from independence expectations.

"Channelling"

The above analysis is clearly relevant to the problem of "channelling" (discussed in Chapters 21 and 22 of this book by Keleti and Ovádi respectively), which deals with the possible effects of certain enzyme-enzyme interactions. The kinetic approach in such publications compares rates calculated from model equations with experimentally obtained values of such rates. Control analysis, on the other hand, asks whether π_i^j and π_j^i are or are not significantly different from 1 and 0 respectively at the operating point of the system. If they are not, then the control distribution is unaffected *even if there is a mechanism that could affect the system under different conditions*. If they do differ from the additivity and independence expectations, such experimentally discovered values could form the basis of other detailed work concerning the mechanism.

It should be noted that the present analysis (as indeed all prior publications on this subject, Kacser & Burns, 1973; 1979; Kacser, 1983; Fell & Sauro, 1985; Sauro *et al.*, 1987; Reder, 1988; Giersch, 1988) are *independent of kinetic mechanisms*. Thus control coefficients, elasticity coefficients and response coefficients are algebraically or operationally defined by partial derivatives or modulation procedures respectively, whatever the kinetic equations of the reactions or those of the system might be. Indeed these coefficients are, in principle, experimentally accessible *without any knowledge of the underlying mechanisms*. Where kinetic formulations are used, they are either by way of example or as the next best thing to their non-mechanistic estimation. The same applies to the π_i^j and π_j^i elasticities discussed in this chapter. They are defined as partial derivatives of the rate, v_i, with respect to E_i or E_j or as responses of the measured isolated rate to changes in these concentrations at the operating point.

This model- and mechanism-independent approach should be contrasted with the attempts to derive relationships based on known (or assumed) kinetic formulations, often of a rather simplified kind. While it would be wrong to dismiss these approaches as providing no insight, they do suffer from the disadvantage of depending (*a*) on complete knowledge of a mechanism and (*b*) on the precision of the parameters used. Both of these may turn out to be unreliable. Many of the controversies between kineticists arise from differences in interpretation of kinetic results, often obtained under different conditions. In contrast, the values of coefficients obtained *in vivo* and used in control analysis are independent of assumptions but suffer, of course, from problems of experimental errors. Both approaches coincide in the necessity to have *structural* information (metabolic map, effector loops, etc.). This constitutes a "model" of some kind and is basic to any analytical approach.

Finally, it must be quite clear that the philosophies underlying the two approaches are

quite different. Kinetic studies attempt to discover mechanism (see, e.g., Ovádi in Chapter 22 of this book), whereas control analysis does not, but attempts to describe quantitatively the control distribution in terms of the responses of the constitutive parts (see also Chapter 17 by Sauro in this book). Much misunderstanding has been generated by a failure to appreciate these fundamental differences.

REFERENCES

Cascante, M., Franco, R. & Canela, E. I. (1989) *Math. Biosci. 94,* 271-288
Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561
Giersch, C. (1988*a*) *Eur. J. Biochem. 174,* 509-513
Giersch, C. (1988*b*) *Eur. J. Biochem. 174,* 515-519
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95
[2]Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Kacser, H. & Burns, J. A. (1979) *Biochem. Soc. Trans. 7,* 1149-1161
Kacser, H. (1983) *Biochem. Soc. Trans. 11,* 35-40
Kacser, H., Sauro, H. M. & Acerenza, L. (1990) *Eur. J. Biochem. 187,* 481-491
Reder, C. (1988) *J. Theor. Biol. 135,* 175-202
Sauro, H. M. & Kacser, H. (1990) *Eur. J. Biochem. 187,* 493-500
Sauro, H. M., Small, J. R. & Fell, D. A. (1987) *Eur. J. Biochem. 165,* 215-221
Savageau, M. A. (1976) *Biochemical Systems Analysis: a Study of Function and Design in Molecular Biology,* Addison-Wesley, Reading, Massachusetts
Srere, P. A. (1987) *Ann. Rev. Biochem. 56,* 21-56
Westerhoff, H. V. & Chen, Y.-D. (1984) *Eur. J. Biochem. 142,* 425-430

[2]EDITORS' NOTE. This publication is often cited as follows: Kacser, H. & Burns, J. A. (1973) in *Rate Control of Biological Processes* (Davies, D. D., ed.), pp. 65-104, Cambridge University Press. In this book we have standardized on the form of citation used by the authors of this chapter.

Chapter 21

Coupled Reactions and Channelling: their Role in the Control of Metabolism

TAMÁS KELETI

CONTROL analysis was developed for describing the regulatory properties of metabolic pathways (Savageau, 1972, 1976; Kacser & Burns, 1973, 1979; Kacser, 1983; Heinrich & Rapoport, 1973, 1974*ab*, 1983; Heinrich *et al.*, 1977). The more effective the control, the more elastic the metabolic pathway, *i.e.* more able to respond to changes in external conditions. Kacser (1983) has suggested the idea of "molecular democracy" to characterize each enzyme in a metabolic process as an autonomous entity and the control as a sort of linear superposition of the effects of the individual enzymes. The milieu of this "molecular society" is a bulk aqueous solution with non-interacting enzymes and non-compartmentalized metabolites homogeneously dispersed therein. The links in such a metabolic network are the intermediate metabolite pools.

However, there is now abundant evidence that the majority of cellular metabolism is spatially organized in membrane-adsorbed enzyme clusters, static and dynamic multienzyme complexes, enzyme arrays attached to the cytomatrix (Masters, 1981; Clegg, 1984; Friedrich, 1984; Welch, 1985; Damjanovich *et al.*, 1986; Welch & Clegg, 1986; Srivastava & Bernhard, 1986, 1987; Srere, 1987; Keleti & Ovádi, 1988; Keleti *et al.*, 1989*a*). Consequently, cell metabolism is more likely controlled by "supramolecular socialism" (Welch & Keleti, 1987) where the "cytosociological" behaviour of the enzymes is manifested in the evolutionarily governed formation of multienzyme systems (Welch & Keleti, 1981).

In our institute several enzyme-pairs catalysing subsequent coupled reactions have been investigated to study the possibility of complex formation, interaction and intermediate channelling. The results are summarized in Table 1. For similar results in other laboratories, we refer to recent reviews (Friedrich, 1984; Srivastava & Bernhard, 1986; Srere, 1987; Keleti & Ovádi, 1988; Keleti *et al.*, 1989*a*). More detail about channelling and its implications may be found in Chapter 22 by Ovádi in this book. To introduce the concept of organized enzyme systems into the original metabolic control analysis defined for non-interacting enzymes in bulk medium, we begin with the treatment of the *kinetic power*.

Tamás Keleti ● Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest H-1502, Hungary ◆ With great sadness, we announce that Tamás Keleti died suddenly on the 4th October 1989

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

Table 1. Methods for detecting complex formation between enzymes

Method	Enzymes	References
Kinetic	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Keleti *et al.* (1977); Ovádi & Keleti (1978)
	Aldolase / glycerophosphate dehydrogenase	Vértessy & Ovádi (1987)
	Aspartate aminotransferase / glutamate dehydrogenase	Salerno *et al.* (1982)
	Phosphofructokinase / fructose bisphosphatase	Ovádi *et al.* (1986)
	Aldolase / phosphofructokinase / calmodulin	Orosz *et al.* (1987, 1988*ab*); Ovádi (1988, 1989)
	2-Phosphoglycerate mutase / γ-enolase	Batke *et al.* (1988)
Thermodynamic	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Keleti (1978)
Chemical	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Patthy & Vas (1978)
	Aldolase / triose phosphate isomerase	Salerno & Ovádi (1982)
Physico-chemical	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Ovádi *et al.* (1978); Batke & Tompa (1986); Tompa *et al.* (1986)
	Aldolase / glycerophosphate dehydrogenase	Batke *et al.* (1980); Ovádi *et al.* (1983)
	Aspartate aminotransferase / glutamate dehydrogenase	Salerno *et al.* (1975)
	Citrate synthase / malate dehydrogenase	Tompa *et al.* (1987)
	2-Phosphoglycerate mutase / γ-enolase	Batke *et al.* (1988)
Isotope-kinetic	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Ovádi (1986); Orosz & Ovádi (1986*ab*, 1987)
	Aldolase / triose phosphate isomerase	Orosz & Ovádi (1986*a*), Orosz *et al.* (1986)
Location near and interaction with membranes	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Solti & Friedrich (1976); Cseke *et al.* (1978); Solti *et al.* (1981)
Compartmentalization of intermediates	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Friedrich *et al.* (1977); Solti & Friedrich (1979)
Pre-lytic protein release	Aldolase / glyceraldehyde 3-phosphate dehydrogenase	Szabolcsi & Cseke (1981) Cseke & Szabolcsi (1983)
Mathematical modelling	Aldolase / glycerophosphate dehydrogenase	Ovádi *et al.* (1985)
Coupled reaction within a single co-crystal	Aldolase / glycerophosphate dehydrogenase	Keleti *et al.* (1989*b*)

The Kinetic Power: a Reappraisal

The rate of the simplest enzyme reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

in which E is the enzyme, S is the substrate, P is the product, and k_1, k_{-1} and k_2 are rate constants, is described by the Michaelis-Menten equation (Michaelis & Menten, 1913; *cf.* Keleti, 1981):

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

where $V_{max}=k_2[E]_T$ is the maximal velocity, $[E]_T$ is the total enzyme concentration and $K_m = (k_{-1} + k_2)/k_1$ is the Michaelis constant. This equation is usually analysed by one of its linearizations, most frequently that of Lineweaver & Burk (1934; *cf.* Keleti, 1986):

$$1/v = (K_m/V_{max})(1/[S]) + 1/V_{max} \quad (2)$$

A straight line in the second quadrant with a positive intercept on the ordinate is characterized by two independent quantities: its intercept on the ordinate and its slope. Unfortunately, for about 50 years the intercept on the ordinate and its ratio with the reciprocal slope (*i. e.* the intercept of the extrapolation of the straight line on the abscissa, equal to $-1/K_m$) were thus considered as independent variables. However, Cleland (1975, 1977) and Northrop (1983) drew attention to the fact that it is V_{max} and V_{max}/K_m that are really the independent kinetic parameters in enzyme action.

In the 1970s k_2/K_m (the apparent second order rate constant), termed the catalytic power or specificity constant, was considered the proper parameter for describing an enzyme's efficiency and thus as the central parameter in enzyme kinetics (Fersht, 1977; Welch & Keleti, 1981). However, we have more recently placed primacy on the metabolic conversion of substrate to product *per se*, within the context of enzymic activity *in situ* . To this end, we have defined the kinetic power, $k_\Gamma = V_{max}/K_m$, which encompasses all factors which bear upon the conversion of free substrate to free product within the cell (Keleti & Welch, 1984; Keleti, 1988). Consequently, the linearized rate equation may be written:

$$1/v = (1/k_\Gamma)(1/[S]) + 1/V_{max} \quad (3)$$

or in the form of the Hanes (1932) plot to have dimensions as time (*cf.* Cornish-Bowden, 1987):

$$[S]/v = 1/k_\Gamma + [S]/V_{max} \quad (4)$$

From the definition of the kinetic power the following result is obtained by taking its reciprocal (Keleti & Welch, 1984):

$$1/k_\Gamma = K_m/V_{max} = K_s/V_{max} + 1/k_1[E_T] \quad (5)$$

where $K_S = k_{-1}/k_1$ is the dissociation constant of the ES complex.

Consequently, k_Γ depends on the equilibrium dissociation constant of the ES complex, the maximal catalytical capacity (V_{max}) and the diffusion-controlled rate constant for ES formation.

Kinetic Power of Allosteric Enzymes

Let us assume an enzyme with a single substrate to be allosteric and to follow the symmetry model (Monod *et al.*, 1965). Since, from eqn. (5)

$$1/k_\Gamma = 1/K_a V_{max} + 1/k_1[E_T] \tag{6}$$

(where K_a is the association constant of ES complex) if $K_{a,i}$ is the association constant of enzyme-substrate complex on the ith subunit (i =1 ... 4 in a tetramer)

$$K_{a,i} = (K_R^i + LK_T^i)/(K_R^{i-1} + LK_T^{i-1}) \tag{7}$$

where K_R is the association constant of the ES complex in the R state and K_T is the same in the T state. L is the allosteric equilibrium constant of the step $E_T \Leftrightarrow E_R$, *i. e.* $L = [E_T]/[E_R]$, where E_R and E_T are the free enzyme in R and T state respectively. Consequently

$$1/k_\Gamma = \{\sum_{i=1}^{n} [(K_R^{i-1} + LK_T^{i-1})/(K_R^i + LK_T^i)]V_{max} + n(1/k_1[E]_T)\}/n \tag{8}$$

In the case of the tetrahedral sequential model (Koshland *et al.*, 1966) assuming the subunits to exist in conformation A in the absence and B in the presence of bound substrate, the association constants of ES are

$$K_{a,1} = K_b K_c K_d^3$$

$$K_{a,2} = K_b K_c K_d K_e$$

$$K_{a,3} = K_b K_c K_e^2 / K_d$$

$$K_{a,4} = K_b K_c K_e^3 / K_d^3 \tag{9}$$

for the four subunits, where K_b is the association constant of S with the B conformation of subunits, K_c is the equilibrium constant of the conversion of the subunit in conformation A to conformation B, K_d is the subunit interaction factor between subunits in forms A and B and K_e that between a pair of B subunits. In this case

$$1/k_\Gamma = [\sum_{i=1}^{n} (1/K_{a,i}V_{max}) + n(1/k_1[E]_T)]/n \tag{10}$$

where $K_{a,i}$ (i=1 ... 4 for a tetramer) are the association constants defined in eqns. (9).

Kinetic Power of Enzymes Catalysing Two-substrate Reactions

Let us assume a simplified general reaction mechanism with two substrates:

$$E + A + B \underset{k_{-1}}{\overset{k_1}{\Longleftrightarrow}} EAB \xrightarrow{k_2} E + P + Q$$

assuming $k_{1,A}$ and $k_{1,B}$ to be the second-order rate constants of the association of EA and EB, respectively, k_2 to be the first-order rate constant of the decomposition of EAB into products and free enzyme. V_{max} is $k_2[E]_T$ and $k_{-1,A}$ and $k_{-1,B}$ are the first-order rate constants of the dissociation of EA and EB, respectively, into free substrates and enzyme. We denote the respective Michaelis and dissociation constants with $K_{m,A}$, $K_{m,B}$, $K_{S,A}$, $K_{S,B}$. In random mechanisms we assume the elementary steps EA+B $\Leftrightarrow$ EAB and EB+A $\Leftrightarrow$ EAB to be very rapid in both directions as compared to the others. In the case of ordered mechanisms $k_{1,B}$ and $k_{-1,B}$ are the association and dissociation rate constants, respectively, of B on the EA complex. In this case the steps E+B $\Leftrightarrow$ EB and EB+A $\Leftrightarrow$ EAB are obviously lacking and the step EA+B $\Leftrightarrow$ EAB is not considered to be very rapid as compared to the others.

We define the kinetic power of the enzyme catalysing the reaction of substrates A and B as:

$$k_{\Gamma,A,B} = V_{max}/[(K_{m,A} + K_{m,B})/2] \tag{11}$$

i. e. the kinetic power is the ratio of maximal velocity and the average Michaelis constant. The individual Michaelis constants are defined as $K_{m,A} = (k_{-1,A} + k_2)/k_{1,A}$, and $K_{m,B} = (k_{-1,B} + k_2)/k_{1,B}$).

From eqn. (11) it follows that:

$$\begin{aligned} k_{\Gamma,A,B} &= V_{max}/[(K_{S,A} + k_2/k_{1,A} + K_{S,B} + k_2/k_{1,B})/2] \\ &= 2k_{1,A}k_{1,B}k_2[E]_T/[k_{1,A}k_{1,B}(K_{S,A} + K_{S,B}) + k_2(k_{1,A} + k_{1,B})] \end{aligned} \tag{12}$$

where $K_{S,A} = k_{-1,A}/k_{1,A}$, $K_{S,B} = k_{-1,B}/k_{1,B}$.

It is obvious that in general case the kinetic power is the function of the ratios of dissociation and decomposition of the central complex and its formation.

$$1/k_{\Gamma,A,B} = (K_{S,A} + K_{S,B})/2k_2[E]_T + (k_{1,A} + k_{1,B})/2k_{1,A}k_{1,B}[E]_T \tag{13}$$

If $k_{1,A} = k_{1,B} = k_D$, where k_D is the diffusion rate constant, then

$$1/k_{\Gamma,A,B} = (K_{S,A} + K_{S,B})/2k_2[E]_T + 1/k_D[E]_T \tag{14}$$

which is identical with the reciprocal of the kinetic power of one-substrate reactions (*cf.* Keleti & Welch, 1984) using average dissociation constant. Eqn. (14) is true if and only if the binding of both substrates to the enzyme is diffusion-limited. We can transform eqn. (14) into

$$1/k_{\Gamma,A,B} = (1/k_D[E]_T)[(K_{-1,A} + K_{-1,B})/2k_2 + 1] \tag{15}$$

and if $k_{-1,A} \approx k_{-1,B} \approx k_2$, which is adequate to the evolutionary "compromise" condition for a simple enzyme function (Knowles, 1976; Brocklehurst, 1977)

$$k_{\Gamma,A,B} = k_D[E]_T/2 \tag{16}$$

Eqn. (16) shows the evolutionary maximum of kinetic power for a two-substrate enzyme in the conditions defined above to be equal to half of the diffusion-controlled rate constant, identically with the evolutionary maximum of kinetic power for a single-substrate enzyme (Keleti & Welch, 1984).

Kinetic Power and Transient Time

The kinetic power is a key parameter not only in the catalysis of individual enzymes but also in the overall behaviour of multienzyme systems. For a two-enzyme coupled reaction such as

$$\begin{matrix} & E_1 & & E_2 & \\ S & \rightarrow & I & \rightarrow & P \end{matrix}$$

where S is the substrate of the first enzyme, I is the intermediate, *i. e.* the product of the first and the substrate of the second enzyme and P is the product of the second enzyme, the steady-state transient time in the general case (Bartha & Keleti, 1969; Easterby, 1981, 1986; Keleti, 1984) is:

$$\tau = ([E_2]_T + K_{m,2})/k_{2,2}[E_2]_T = 1/k_{2,2} + 1/k_{\Gamma,2} \tag{17}$$

where $k_{2,2}$, $K_{m,2}$ and $1/k_{\Gamma,2}$ are the first-order rate constant of product formation, the Michaelis constant and the kinetic power for the second enzyme, respectively. In the limiting case when $[E_2]_T \ll K_{m,2}$ (Hess & Wurster, 1970; Easterby, 1973; Welch, 1977*b*), this reduces to

$$\tau = K_{m,2}/k_{2,2}[E_2]_T = 1/k_{\Gamma,2} \tag{18}$$

(Keleti & Welch, 1984; Keleti & Vértessy, 1986; Welch *et al.*, 1988).

For a sequence of n irreversible enzyme reactions rate-limited by an initial governing step with the subsequent enzyme steps subsaturated ($[S_i] \ll K_{m,i}$):

$$\tau = \sum_{i=1}^{n} \tau_i = \sum_{i=1}^{n} 1/k_{\Gamma,i} \tag{19}$$

(Welch *et al.*, 1988).

The smaller the transient time, the more efficient the metabolic pathway of which the given enzymes are part.

Control in Homogeneous Enzyme Systems

Assuming a chain of unsaturated but reversible enzymes carrying out the overall conversion of external substrate X_1 to an external product X_2 *via* successive intermediary metabolites

$S_1, S_2, \ldots S_{n-1}$

$$\begin{matrix} & E_1 & & E_2 & & & & E_n \\ X_1 & \rightarrow & S_1 & \rightarrow & S_2 & \rightarrow \cdots & S_{n-1} & \rightarrow X_2 \end{matrix}$$

it follows (Kacser & Burns, 1973) by using the term of kinetic power (Keleti & Vértessy, 1986, Welch *et al.*, 1988) that the overall flux, F, for this system is:

$$F = ([X_1] - [X_2]/K_{eq,1}K_{eq,2}\cdots K_{eq,n})/(1/k_{\Gamma,1} + 1/k_{\Gamma,2}K_{eq,1} + 1/k_{\Gamma,3}K_{eq,1}K_{eq,2} + \cdots) \quad (20)$$

where $K_{eq,1}$ is the equilibrium constant between pools X_1 and S_1, $K_{eq,2}$ is that between pools S_1 and S_2, etc.

The factor describing the response of the overall rate to an infinitesimal change in enzyme concentration is the control coefficient (Kacser & Burns, 1973, Heinrich & Rapoport, 1974*a*). We suggest that the kinetic power is the most appropriate variable for this coefficient (Keleti & Vértessy, 1986, Cornish-Bowden, 1987, Welch *et al.*, 1988).We can therefore define the coefficient as:

$$C_\Gamma^F = \frac{(\partial F/F)}{(\partial k_\Gamma/k_\Gamma)} = (k_\Gamma/F)\frac{\partial F}{\partial k_\Gamma} \quad (21)$$

Following the procedures of Kacser & Burns (1973, 1979) but using the kinetic power as variable (Keleti & Vértessy, 1986; Welch *et al.*, 1988), we find for the system in Scheme 1:

$$C_{\Gamma,1}^F = (1/k_{\Gamma,1})/D$$

$$C_{\Gamma,2}^F = (1/k_{\Gamma,2}K_{eq,1})/D$$

$$C_{\Gamma,3}^F = (1/k_{\Gamma,3}K_{eq,1}K_{eq,2})/D \quad (22)$$

where

$$D = 1/k_{\Gamma,1} + 1/k_{\Gamma,2}K_{eq,1} + 1/k_{\Gamma,3}K_{eq,1}K_{eq,2} + \cdots \quad (23)$$

The elasticity coefficient is defined (Kacser & Burns, 1973, 1979; Heinrich & Rapoport, 1974*a*; Heinrich *et al.*, 1977) as follows:

$$\varepsilon_S^v = \frac{\partial v/v}{\partial S/S} = (S/v)\left(\frac{\partial v}{\partial S}\right) \quad (24)$$

where v is the velocity of the enzyme reaction and S is the concentration of substrate.

In the case of Michaelis-Menten kinetics (single enzyme-substrate complex and rate-determining irreversible elementary step of product formation)

$$\varepsilon_S^v = K_m/(K_m + [S]) = V_{max}/(V_{max} + k_\Gamma[S]) \quad (25)$$

(Crabtree & Newsholme, 1985, Keleti & Vértessy, 1986).

For a reversible Michaelis-Menten enzyme reaction of the form:

$$E + S \Leftrightarrow ES \Leftrightarrow EP \Leftrightarrow E + P$$

the elasticity coefficient, by using the kinetic power as variable, will have the following form (Westerhoff *et al.*, 1984; Welch *et al.*, 1988):

$$\varepsilon_S^v = k_\Gamma^f[S]/(k_\Gamma^f[S] - k_\Gamma^r[P]) - k_\Gamma^f[S]/\{V_{max}^f + k_\Gamma^f[S] + k_\Gamma^r[P](V_{max}^f/V_{max}^r)\} \quad (26)$$

where k_Γ^f, k_Γ^r, V_{max}^f, V_{max}^r are the kinetic power and maximal velocity, respectively, for the forward (f) and reverse (r) directions.

Control in Heterogeneous Enzyme Systems

The structural flux-control coefficient for an organized multienzyme system (Welch *et al.*, 1988) is as follows:

$$C_{\Gamma,s}^F = \sum_{r=1}^{m} \beta_{rs}\left(\frac{\partial \ln F}{\partial \ln \pi_r}\right) \quad (27)$$

where $$\pi_r = \prod_{i=1}^{n_r} \alpha_{i,r} \quad \text{and} \quad \beta_{rs} = \frac{\partial \ln \pi_r}{\partial \ln \pi_s}$$

The $\alpha_{i,r}$ denote all (dimensionless) extrinsic factors arising in the organized state (Keleti, 1975; Welch, 1977*a*; Welch & Keleti, 1981).

We consider that the system is composed of m enzyme-catalysed reactions, and n_r are the number of factors bearing upon the kinetic power of the rth reaction. In this case the kinetic power is:

$$k_{\Gamma,r} = k_{\Gamma,r}^* \prod_{i=1}^{n_r} \alpha_{i,r} \quad (r = 1, 2, \cdots m) \quad (28)$$

where $k^*_{\Gamma,r}$ is the intrinsic kinetic power (Welch *et al.*, 1988).

In the case of dynamically interacting systems we do not know whether the given enzyme system is physically organized in the cell. Thus, we must write an apparent control coefficient (Welch *et al.*, 1988):

$$C_\Gamma^{F(app)} = (k_\Gamma^c/F^{(app)})\left(\frac{\partial F^{(app)}}{\partial k_\Gamma^c}\right) + (k_\Gamma^b/F^{(app)})\left(\frac{\partial F^{(app)}}{\partial k_\Gamma^b}\right) \quad (29)$$

where superscripts c and b denote the organized (channelled) and bulk-solution components of flux control, respectively.

Control of Metabolism in "Near-equilibrium"

If the catalytic capacity of an enzyme in the cell is much higher than that of the other enzymes which react with the same substrates, then it will bring its own reaction partners very close to thermodynamic equilibrium. This is called "near-equilibrium relation" (Holzer *et al.*, 1956; Bücher & Klingenberg, 1958; Hohorst *et al.*, 1959; Veech *et al.*, 1969; Krebs & Veech, 1969; Veech *et al.*, 1970). Since the kinetic requirements for a near-equilibrium relation are not too restrictive it can be expected that whole pathways may consist of them (Reich, 1976). If we assume the whole metabolic pathway in near-equilibrium, the K_{eq} values defined originally as equilibrium constant between their pools will be simply the thermodynamic equilibrium constants of the reversible reactions catalysed by the corresponding enzymes E_i. Since K_{eq} according to the Haldane relation is equal to k_Γ^f/k_Γ^r (Keleti & Welch, 1984), we will have for the equation of the flux:

$$F = ([X_1]\prod_{i=1}^{n} k_{\Gamma,i}^{f} - [X_2]\prod_{j=1}^{n} k_{\Gamma,j}^{r})/D_c \tag{30}$$

where

$$D_c = \prod_{i=2}^{n} k_{\Gamma,i}^{f} + k_{\Gamma,1}^{r}\prod_{i=3}^{n} k_{\Gamma,i}^{f} + \prod_{j=1}^{2} k_{\Gamma,j}^{r}\prod_{i=4}^{n} k_{\Gamma,i}^{f} + \prod_{j=1}^{3} k_{\Gamma,j}^{r}\prod_{i=5}^{n} k_{\Gamma,i}^{f} + \cdots + \prod_{j=1}^{n-1} k_{\Gamma,j}^{r} \tag{31}$$

The use of the kinetic power reveals the specific feature of the parameters of metabolic control even in near-equilibrium conditions. The flux depends only on the concentration of the first substrate and last product in the whole pathway and on the kinetic power of all enzymes. The control coefficients depend only on the kinetic powers (Keleti, 1989).

Conclusion

Using the kinetic power as key parameter all equations and parameters of the metabolic control receive a simple form independently of whether the metabolic pathway takes part in homogeneous bulk medium with non-interacting enzymes or in heterogeneous interacting enzyme systems. The equations reflect the velocities of the enzymes, the flux of the whole pathway, the parameters of the metabolic control if the flux governs the metabolic pathway or if the metabolic pathway is in "near-equilibrium".

Since $1/k_\Gamma$ for the second enzyme of a coupled system is equal to τ, the transient time of the coupled reaction (Keleti & Vértessy, 1986; Welch *et al.*, 1988; Keleti & Ovádi, 1988; Keleti, 1988; Ovádi *et al.*, 1989) all reactions that reflect $1/k_\Gamma$ of the second enzyme of a coupled reaction is relevant for the transient time. This means that the reciprocal of kinetic power reflects the transient time for a second enzyme of Michaelis-Menten type, for an allosteric enzyme that follows the Monod-Wyman-Changeux or Koshland-Némethy-Filmer model, as well as for enzymes with two substrates.

REFERENCES

Bartha, F. & Keleti, T. (1979) *Oxid. Commun. 1*, 75-84

Batke, J. & Tompa, P. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, S., Keleti, T. & Trón, L. eds.). Akadémiai Kiadó, Budapest, and Elsevier, Amsterdam. pp. 247-266

Batke, J., Asbóth, G., Lakatos, S., Schmitt, B. & Cohen, R. (1980) *Eur. J. Biochem. 107*, 389-394

Batke, J., Nazaryan, K. B. & Karapetian, N. H. (1988) *Arch. Biochem. Biophys. 264*, 510-518

Brocklehurst, K. (1977) *Biochem. J. 163*, 111-116

Bücher, T. & Klingenberg, M. (1958) *Angew. Chem. 70*, 552-570

Clegg, J. S. (1984) *Am. J. Physiol. 246*, R133-R151

Cleland, W. W. (1975) *Acc. Chem. Res. 8*, 145-151

Cleland, W. W. (1977) *Adv. Enzymol. 45*, 273-387

Cornish-Bowden, A. (1987) *Biochem. Educ. 15*, 144-146

Crabtree, B. & Newsholme, E. A. (1985) *Curr. Top. Cell. Regul. 25*, 21-76

Cseke, E. & Szabolcsi, G. (1983) *Acta Biochim. Biophys. Acad. Sci. Hung. 18*, 151-161

Cseke, E., Váradi, A., Szabolcsi, G. & Biszku, E. (1978) *FEBS Lett. 96*, 15-18

Damjanovich, S., Keleti, T. & Trón, L. (eds.) (1986) *Dynamics of Biochemical Systems*, Akadémiai Kiadó, Budapest, and Elsevier, Amsterdam

Easterby, J. S. (1973) *Biochim. Biophys. Acta 293*, 552-558

Easterby, J. S. (1981) *Biochem. J. 199*, 155-161

Easterby, J. S. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, S., Keleti, T. & Trón, L., eds.), Akadémiai Kiadó, Budapest and Elsevier, Amsterdam, pp. 145-158

Fersht, A. (1977) *Enzyme Structure and Mechanism*. Freeman, San Francisco

Friedrich, P. (1984) *Supramolecular Enzyme Organization. Quaternary Structure and Beyond*, Pergamon Press, Oxford, and Akadémiai Kiadó, Budapest

Friedrich, P., Apró-Kovács, A. V. & Solti, M. (1977) *FEBS Lett. 84*, 183-186

Hanes, C. S. (1932) *Biochem. J. 26*, 1406-1421

Heinrich, R. & Rapoport, S. M. (1983) *Biochem. Soc. Trans. 11*, 31-35

Heinrich, R. & Rapoport, T. A. (1973) *Acta Biol. Med. Germ. 31*, 479-494

Heinrich, R. & Rapoport, T. A. (1974*a*) *Eur. J. Biochem. 42*, 89-95

Heinrich, R. & Rapoport, T. A. (1974*b*) *Eur. J. Biochem. 42*, 97-105

Heinrich, R., Rapoport, S. M. & Rapoport, T. A. (1977) *Progr. Biophys. Molec. Biol. 32*, 1-82

Hess, B. & Wurster, B. (1970) *FEBS Lett. 9*, 73-77

Hohorst, H., Kreutz, F. H. & Bücher, T. (1959) *Biochem. Z. 332*, 18-46

Holzer, H., Schultz, G. & Lynen, F. (1956) *Biochem. Z. 328*, 252-263

Kacser, H. (1983) *Biochem. Soc. Trans. 11*, 35-60

Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27*, 65-104

Kacser, H. & Burns, J. A. (1979) *Biochem. Soc. Trans. 7*, 1149-1160

Keleti, T. (1975) in *Mechanism of Action and Regulation of Enzymes* (Keleti, T., ed.) Akadémiai Kiadó, Budapest, and North-Holland, Amsterdam. pp. 3-27

Keleti, T. (1978) in *New Trends in the Description of the General Mechanism and Regulation of Enzymes* (Damjanovich, S., Elödi, P. & Somogyi, B., eds.) pp. 107-130, Akadémiai Kiadó, Budapest

Keleti, T. (1981) in *Kinetic Data Analysis* (Endrényi, L., ed.) Plenum Press, New York, pp. 353-374

Keleti, T. (1984) in *Dynamics of Biochemical Systems* (Ricard, J. & Cornish-Bowden, A., eds.) pp. 103-114, Plenum Press, New York

Keleti, T. (1986) *Basic Enzyme Kinetics*, Akadémiai Kiadó, Budapest

Keleti, T. (1988) *J. Mol. Catal. 47*, 271-279

Keleti, T. (1989) *Nova Acta Leopoldina* in press

Keleti, T. & Ovádi, J. (1988) *Curr. Top. Cell. Regul. 29*, 1-33

Keleti, T. & Vértessy, B. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, S., Keleti, T. & Trón, L., eds.) pp. 3-10, Akadémiai Kiadó, Budapest, and Elsevier, Amsterdam

Keleti, T. & Welch, G. R. (1984) *Biochem. J. 223,* 299-303
Keleti, T., Batke, J., Ovádi, J., Jancsik, V. & Bartha, F. (1977) *Adv. Enzyme Regul. 15,* 233-265
Keleti, T., Ovádi, J. & Batke, J. (1989*a*) *Progr. Biophys. Mol. Biol. 53,* 105-152
Keleti, T., Berni, R., Vas, M., Mozzarelli, A. & Rossi, G. L. (1989*b*) *Acta Biochim. Biophys. Hung. 24,* 15-23
Knowles, J. R. (1976) *FEBS Lett. 62* (Suppl.), E53-E61
Koshland, D. E., Jr., Némethy, G. & Filmer, D. (1966) *Biochemistry 5,* 365-385
Krebs, H. A. & Veech, R. L. (1969) *Adv. Enzyme Regul. 7,* 397-413
Lineweaver, H., Burk, D. (1934) *J. Am. Chem. Soc. 56,* 658-666
Masters, C. J. (1981) *CRC Crit. Rev. Biochem. 11,* 105-144
Michaelis, L. & Menten, M. L. (1913) *Biochem. Z. 49,* 333-369
Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol. 12,* 88-118
Northrop, D. B. (1983) *Anal. Biochem. 132,* 457-461
Orosz, F. & Ovádi, J. (1986*a*) *Eur. J. Biochem. 160,* 615-619
Orosz, F. & Ovádi, J. (1986*b*) in *Chromatography '84* (Kalász, H. & Ettre, L., eds.) Elsevier, Amsterdam, and Akadémiai Kiadó, Budapest. pp. 425-434
Orosz, F. & Ovádi, J. (1987) *Biochim. Biophys. Acta 915,* 53-59
Orosz, F., Nuridsány, M. & Ovádi, J. (1986) *J. Biochem. Biophys. Meth. 13,* 325-332
Orosz, F., Christova, T. Y. & Ovádi, J. (1987) *Biochem. Biophys. Res. Commun. 147,* 1121-1128
Orosz, F., Christova, T. Y. & Ovádi, J. (1988*a*) *Biochim. Biophys. Acta 95,* 293-300
Orosz, F., Christova, T. Y. & Ovádi, J. (1988*b*) *Mol. Pharmacol. 33,* 678-682
Ovádi, J. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, S., Keleti, T. & Trón, L., eds.). Akadémiai Kiadó, Budapest, and Elsevier, Amsterdam, pp. 203-216
Ovádi, J. (1988) *Trends Biochem. Sci. 13,* 486-490
Ovádi, J. (1989) *Progr. Drug Res. 33,* 353-395
Ovádi, J. & Keleti, T. (1978) *Eur. J. Biochem. 85,* 157-161
Ovádi, J., Salerno, C., Keleti, T. & Fasella, P. (1978) *Eur. J. Biochem. 90,* 499-503
Ovádi, J., Mohamed Osman, I. R. & Batke, J. (1983) *Eur. J. Biochem. 133,* 433-437
Ovádi, J., Mátrai, Gy., Bartha, F. & Batke, J. (1985) *Biochem. J. 229,* 57-62
Ovádi, J., Aragón, J. J. & Sols, A. (1986) *Biochem. Biophys. Res. Commun. 135,* 852-856
Ovádi, J., Tompa, P., Vértessy, B., Orosz, F., Keleti, T. & Welch, G. R. (1989) *Biochem. J. 257,* 187-190
Patthy, L. & Vas, M. (1978) *Nature 276,* 94-95
Reich, J. G. (1976) in *Mathematical Models of Metabolic Regulation* (Keleti, T. & Lakatos, S., eds.). Akadémiai Kiadó, Budapest, pp. 159-171
Salerno, C. & Ovádi, J. (1982) *FEBS Lett. 138,* 270-272
Salerno, C., Ovádi, J., Churchich, J. & Fasella, P. (1975) in *Mechanism of Action and Regulation of Enzymes* (Keleti, T., ed.) Akadémiai Kiadó, Budapest and North-Holland, Amsterdam, pp. 147-160
Salerno, C., Ovádi, J., Keleti, T. & Fasella, P. (1982) *Eur. J. Biochem. 121,* 511-517
Savageau, M. A. (1972) *Curr. Top. Cell. Regul. 6,* 63-130
Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology,* Addison-Wesley, Reading, Massachusetts
Solti, M. & Friedrich, P. (1976) *Mol. Cell. Biochem. 10,* 145-152
Solti, M. & Friedrich, P. (1979) *Eur. J. Biochem. 95,* 551-559
Solti, M., Bartha, F., Halász, N., Tóth, G., Sirokmán, F. & Friedrich, P. (1981) *J. Biol. Chem. 256,* 9260-9265
Srere, P. A. (1987) *Ann. Rev. Biochem. 56,* 21-56
Srivastava, D. K. &Bernhard, S. A. (1986) *Curr. Top. Cell. Regul. 28,* 1-68
Srivastava, D. K. & Bernhard, S. A. (1987) *Ann. Rev. Biophys. Biophys. Chem. 16,* 175-204
Szabolcsi, G. & Cseke, E. (1981) *Acta Biol. Med. Germ. 40,* 471-477
Tompa, P., Bär, J. & Batke, J. (1986) *Eur. J. Biochem. 159,* 117-124
Tompa, P., Batke, J., Ovádi, J., Welch, G. R. & Srere, P. A. (1987) *J. Biol. Chem. 262,* 6089-6092

Veech, R. L., Eggleston, L. V. & Krebs, H. A. (1969) *Biochem. J. 115*, 609-619
Veech, R. L., Raijman, L. & Krebs, H. A. (1970) *Biochem. J. 117*, 499-503
Vértessy, B. & Ovádi, J. (1987) *Eur. J. Biochem. 164*, 655-659
Welch, G. R. (1977*a*) *Progr. Biophys. Molec. Biol. 32*, 103-191
Welch, G. R. (1977*b*) *J. Theor. Biol. 68*, 267-291
Welch, G. R. (ed.) (1985) *Organized Multienzyme Systems: Catalytic Properties*, Academic Press, New York
Welch, G. R. & Clegg, J. S. (eds.) (1986) *The Organization of Cell Metabolism*, Plenum Press, New York
Welch, G. R. & Keleti, T. (1981) *J. Theor. Biol. 93*, 701-735
Welch, G. R. & Keleti, T. (1987) *Trends Biochem. Sci. 12*, 216-217
Welch, G. R., Keleti, T. & Vértessy, B. (1988) *J. Theor. Biol. 130*, 407-422
Westerhoff, H. V., Groen, A. K. & Wanders, R. J. A. (1984) *Biosci. Rep. 4*, 1-22

Chapter 22

Channelling and Channel Efficiency: Theory and Analytical Implications

JUDIT OVÁDI

CHANNELLING of metabolites in a reaction sequence is accepted as the phenomenon where the reaction product of one enzyme is transferred directly to another enzyme as its substrate *via* transient enzyme-enzyme interactions (Srivastava & Bernhard, 1986, 1987). According to the Srivastava-Bernhard hypothesis such a "one-encounter-type metabolite transfer" can occur if the heterologous enzyme complex associates and dissociates during every catalytic turnover of enzymes. Alternatively, an intermediate can be also channelled if its liberation from the active site of one enzyme is followed by direct transfer to the active site of the next enzyme in the sequence without diffusing into the bulk solution (Srere, 1987; Welch, 1985, Keleti & Ovádi, 1988, and references therein; see also Chapter 21 by Keleti in this book). Both mechanisms of intermediate transfer may result in physiological advantages to an organism, such as (i) segregation of competing pathways by microcompartmentation of intermediates, (ii) reduction of the time required to reach the steady state, and (iii) enhancement of metabolite flux by providing high local metabolite concentrations. A new description of the channelling effect has been elaborated (Tompa *et al.*, 1987) based on inherent parameters such as channel efficiency and intermediate lifetime. These inherent parameters, together with the analytical implications, will be discussed in this chapter. In addition, some examples will be presented to illustrate how the mechanism of intermediate transfer in interacting enzyme systems can be determined and how dynamic channelling complexes of enzymes can be specifically modulated.

Theoretical Considerations

Channelling of metabolites is often suggested to play an important role in metabolic economy and efficiency. The kinetic method for its detection and analysis is based upon measuring the time required for attaining the steady-state flux in consecutive reactions catalysed by functionally interacting enzymes (Hess & Wurster, 1970; Easterby, 1981, 1984;

Judit Ovádi ● Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest H-1502, Hungary

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

Keleti & Ovádi, 1988, and references therein; see also Chapter 23 by Easterby in this book). This transient time deduced from the steady-state flux can be expressed as a function of the lifetime of the intermediate substrate; thus its reduction can be explained even if no changes in the kinetic parameters of the individual reactions occur and no physical barrier prevents the diffusion of intermediate into the bulk phase (Tompa *et al.*, 1987).

If the enzymes E_1 and E_2 catalyse consecutive conversion of the initial substrate S to the final product P *via* formation of intermediate I, then the lifetime of the intermediate includes the times required for release from E_1, diffusion, association to and conversion by E_2. The sum of these times for all molecules yields a characteristic lifetime of the whole population. Obviously, the fraction α of molecules that will be converted within E_1E_2 enzyme complex has a shorter lifetime ($\langle t' \rangle$) than that of the non-channelled molecules ($\langle t \rangle$), since the average distance between active centres is shorter within a heterologous enzyme complex than between separated enzyme molecules. The channel efficiency α can be defined as the probability that an intermediate is converted within the generating complex. If the generation of the intermediate proceeds at a constant rate v and E_2 is subsaturated by I, then the concentration of I at steady-state $[I_{ss}]$ for partially complexed enzyme systems is as follows (Tompa *et al.*, 1987):

$$[I_{ss}] = v/[E_1]_{total}\{\alpha[Complex]\langle t' \rangle + ([E_1]_{free} + \{1 - \alpha\}[Complex])\langle t \rangle\}$$

Since $[P] = vt - [I_{ss}]$ at steady-state by extrapolating the linear part of the progress curve of product formation, its intercept on the time axis is the apparent transient time (τ_{app}), which is characteristic for channelling effect in a certain partially complexed enzyme system:

$$\tau_{app} = 1/[E_1]_{total}\{\alpha[Complex]\langle t' \rangle + ([E_1]_{free} + \{1 - \alpha\}[Complex])\langle t \rangle\}$$

where $\langle t \rangle$ is a concentration-dependent parameter ($K_{m,2}/k_{cat,2}[E_2]$), whereas $\langle t' \rangle$ is concentration independent, being an inherent property of the enzyme complexes. By measuring τ_{app} at various enzyme concentrations to ensure different degree of complexation, the channel efficiency and lifetime of the channelled intermediates can be experimentally determined. In general, α varies between 0 and 1, such that $\langle t' \rangle < \tau_{app} < \langle t \rangle$

The decrease in transient time in interacting enzyme system may be due either to the presence of physical barrier on the out-diffusion of intermediate or to the mere juxtaposition of the sequential active sites. Any alteration in the interacting system relating either to the diffusional process or to the kinetic parameters, will manifest in changes of α, $\langle t' \rangle$ and $\langle t \rangle$.

For the determination of these inherent parameters the τ_{app} value should be determined from the steady-state velocity of the coupled reaction at various enzyme concentrations ensuring different degree of complexation and the linearized formula can be used (cf. Tompa *et al.*, 1987). To illustrate the applicability of the approach the experimental data for the aspartate aminotransferase/glutamate dehydrogenase interacting system are shown in Fig.1 together with the model used for calculation of theoretical curves (Fig. 1A). Fig. 1C shows that the transformation of Fig. 1B yields points conforming to a straight line only if an appropriate dissociation constant is employed.

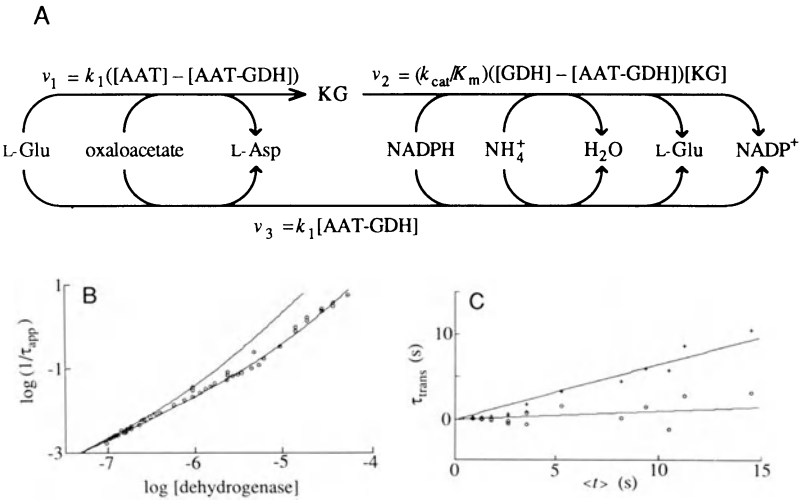


Figure 1. Modelling of the aspartate aminotransferase (AAT) / glutamate dehydrogenase (GDH) interacting system. **A**. Reaction scheme, in which KG = α-ketoglutarate. **B**. Relation between the reciprocal transient time, τ (s) and the concentration of glutamate dehydrogenase (M). The solutions of enzymes at a constant ratio of aspartate aminotransferase/glutamate dehydrogenase 0.088 preincubated for 30 min in EDTA, 0.05M Tris/HCl, pH 7.5, at 25 °C were mixed. The theoretical curve was computed assuming the model with the following parameters: (a) $K_{m,GDH}$ = 0.8 mM, k_{cat} = 17 s^{-1}, K_d = 8.6 μM ; (b) the same except K_d = 0.86 μM. For other experimental conditions see Salerno *et al.* (1982). **C**. Linearization of transient time, τ_{app} for the aspartate aminotransferase / glutamate dehydrogenase system according to the following equation: $\{\tau_{app}[E_1]_{total} - [E_1]_{free}\}/[AAT\text{-}GDH] = \alpha\langle t'\rangle + (1 - \alpha)\langle t\rangle$. Data are taken from Fig.1B, assuming K_d = 8.6 μM (o) or K_d = 0.86 μM (+).

For the study of enzyme-enzyme interactions most of the studies required measurements of kinetic parameters as functions of enzyme concentration. Even if it can be done in a wide range of protein concentration the subunit-subunit interactions of oligomeric enzymes at relatively high concentrations may perturb the heterologous interactions (Keleti *et al.*, 1977). Our simple kinetic approach, which is suitable for identifying the mechanism of intermediate transfer in interacting enzyme systems, overcomes these difficulties (Ovádi, 1986; Orosz *et al.*, 1988; Ovádi *et al.*, 1989). The approach is based on the comparison of macroscopic kinetic parameters determined under interacting and non-interacting conditions. The relation of the transient time τ_{app} for the coupled reaction and the pseudo-first-order rate constants measured in the absence (k_{E_2}) and presence of E_1 ($k_{E_2(E_1)}$) are indicative of the mechanism in interacting enzyme system (cf.Table 1). If the interaction of the two enzymes induces alterations in the ternary or quaternary structure of the enzymes without producing channelling of the intermediate the relationship described in line 2 of Table 1 is fulfilled. If the intermediate produced endogenously by the E_1E_2 complex is channelled between the two enzymes the transient time is reduced with respect to that measured in a non-interacting system. This relationship may result from either steric hindrance which impedes the diffusion of intermediate into the bulk solution (Table 1, line 4) or simply from the juxta-position of active sites of the enzymes in complexed form (Table 1, line 3). The latter case,

Table 1. Relationships of the microscopic and macroscopic kinetic parameters in enzyme systems

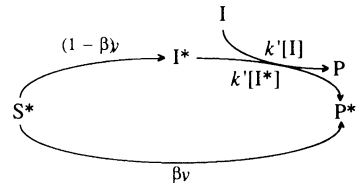
Type of interaction	Macroscopic parameters E_1	E_2	Microscopic parameters	Examples[1]
Non-interacting	$v_{E_1} = v$	$1/\tau_{app} = k_{E_2(E_1)} = k_{E_2}$	$\alpha = 0; \langle t' \rangle = 0$	GAPDH / TPI
Interaction with conformational changes	$v_{E_1} > v$	$1/\tau_{app} = k_{E_2(E_1)} > k_{E_2}$	$0 < \alpha < 1; \langle t' \rangle < \langle t \rangle$	PFK / FBPase
Leaky channel	$v_{E_1} = v$	$1/\tau_{app} > k_{E_2(E_1)} = k_{E_2}$	$0 < \alpha < 1; \langle t' \rangle < \langle t \rangle$	Aldolase / GAPDH
Partial channel	$v_{E_1} = v$	$1/\tau_{app} > k_{E_2} > k_{E_2(E_1)}$	$0 < \alpha < 1; \langle t' \rangle < \langle t \rangle$	Aldolase / GPDH
Perfect channel	$v_{E_1} = v$	$1/\tau_{app} \to \infty;\ k_{E_2(E_1)} \to 0$	$\alpha = 1; \langle t' \rangle = 0$	AAT / GluDH

[1]*Abbreviations:* GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TPI, triose phosphate isomerase; PFK, phosphofructokinase; FBPase, fructose 1,6-bisphosphatase; GPDH, glycerol phosphate dehydrogenase; AAT, aspartate aminotransferase; GluDH, glutamate dehydrogenase

which can be considered as a special one, we denoted leaky channel. If the escape of the intermediate from the complex is prevented the mechanism is denoted as a perfect channel (Table 1, line 5) where $\alpha = 1$ and $\langle t' \rangle = 0$.

If this kinetic approach is combined with the isotope dilution technique the sensitivity of the identification of the mechanism of intermediate transfer is enormously enhanced. In this special experiment the initial substrate of the consecutive reactions is radioactive (S*) and unlabelled intermediate is added to the reaction mixture. The relative specific radioactivity r of the end product P* at a given time t is measured and compared with those calculated according to the various mechanisms (lines 1-5 of Table 1) (Orosz & Ovádi, 1987).

Scheme 1. Use of radioactive label (*) to measure the extent of channelling from a substrate S to a product P via an intermediate I* that may be able to exchange with unlabelled intermediate I.



In dynamically interacting enzyme systems the kinetic parameters one measures are composite functions of those for the processes catalysed by the complexed and by the separated enzymes. The use of the aforementioned, however, requires no knowledge of the dissociation constant of enzyme complex for identifying the mechanism of interaction since the enzyme concentrations used experimentally are the same independently of whether coupled or separate reactions are analysed. However, if the dissociation constant of the enzyme complex is known, then the extent of the channel can be deduced by applying the following equations derived for Scheme 1:

$$r_{channel} = \frac{[P^*]}{[P^*]+[P]} = \frac{vt - (1-\beta)v/[k'(1-e^{-k't})]}{vt - (1-\beta)v/[k'(1-e^{-k't})] + [I_0](1-e^{-k't})}$$

where $\beta = [E_1]_{bound}/[E_1]_{total}$ and $k' = k[E_2]_{free}/[E_2]_{total}$. Therefore, in a dynamically complexed enzyme system the value of the measured relative specific radioactivity is characteristic of the extent, or perfectness, of the "channel", since

$$r_{\text{non-interacting}} \leq r_{\text{measured}} \leq r_{\text{channel}}$$

Experimental Analysis of Intermediate Channelling

These kinetic approaches have been used to identify the mechanism of intermediate transfer in aldolase/glycerol phosphate dehydrogenase and aldolase/glyceraldehyde 3-phosphate dehydrogenase enzyme systems where the complex formation between the enzyme couples has been demonstrated (Vértessy & Ovádi, 1987; Orosz & Ovádi, 1987). The relationships of the kinetic parameters, given in line 4 of Table 1, indicate that the binding of exogenous intermediate (dihydroxyacetone phosphate) to the glycerol phosphate dehydrogenase is impeded probably due to steric hindrance, whereas the endogenous intermediate produced by aldolase has direct access to the dehydrogenase within the complex. In fact, we have found that the pseudo-first-order rate constant measured in the presence of non-functioning aldolase is lower than that measured in the absence of aldolase due to an apparent increase in K_m value of exogenous intermediate for dehydrogenase (Vértessy & Ovádi, 1987). Very recently Chock & Gutfreund (1988) reported that the aldolase is an inhibitor for the dehydrogenase-catalysed reaction since the apparent K_m of dihydroxyacetone phosphate is increased in the presence of aldolase. However, they interpreted these results in a different way; the increase was attributed to the binding of dihydroxyacetone phosphate to aldolase which reduced the free concentration of the triose phosphate. Unfortunately, these authors did not indicate what concentrations of aldolase and substrate were applied in their experiments. Nevertheless, since the apparent K_m value for glycerol phosphate dehydrogenase is of the order of mM, an aldolase concentration comparable to this value should have been applied to have an observable effect on the apparent K_m value due to its triose phosphate binding. Such a high enzyme concentration is hardly realizable in practice. In our experiments the concentration of aldolase was 15 μM or lower; therefore, the segregation of the substrate by non-functioning aldolase could be excluded. The idea of an active-site-directed interaction between aldolase and glycerol phosphate dehydrogenase is consonant with the suggestion of Srivastava & Bernhard (1986), who observed direct transfer of the intermediate substrate from aldolase to glycerol phosphate dehydrogenase using different kinetic approaches.

Another system that has been the subject of detailed investigations in several laboratories is the aldolase/glyceraldehyde 3-phosphate dehydrogenase system (Ovádi & Keleti, 1978; Ovádi *et al.*, 1978; Patthy & Vas, 1978; Kvassman *et al.*, 1988; Grazi & Trombetta, 1980; Orosz & Ovádi, 1987). We have found that the exogenous intermediate, glyceraldehyde 3-phosphate has the same probability to bind to the free dehydrogenase as to the complexed one (cf. line 3 of Table 1). However, the endogenous aldehyde form of glyceraldehyde 3-phosphate liberated at the active site of aldolase is transferred directly, at

least partially, within the heterologous enzyme complex due to the proximity effect. Thus the hydration in the bulk medium of the aldehyde form generated by aldolase before it reaches the active site of glyceraldehyde 3-phosphate dehydrogenase is prevented. Recent data of Kvassman *et al.* (1988) were consistent with ours reported ten years earlier. However, the result of their theoretical analysis have been interpreted to be compatible with a free-diffusion mechanism for the transfer of glyceraldehyde 3-phosphate. In fact, the rate of the enzymatic conversion of glyceraldehyde 3-phosphate in their experiments was much higher than the hydration rate of aldehyde form of glyceraldehyde 3-phosphate since they applied a high excess of dehydrogenase. Under our experimental conditions the rate of the enzymatic reaction catalysed by the dehydrogenase was slower than that of the aldehyde-diol interconversion, therefore, the unfavourable aldehyde-diol conversion of glyceraldehyde 3-phosphate, at least partly, would have occurred if the substrate were to mix with the bulk medium. The channelling of aldehyde form of triose phosphate in the coupled reaction catalysed by aldolase and glyceraldehyde 3-phosphate dehydrogenase has been clearly demonstrated using the isotope dilution technique as well (Orosz & Ovádi, 1987). Moreover, the precise mechanism of intermediate transfer could be identified as a "leaky channel", which refers to a situation when the exogenous intermediate binds to the complexed dehydrogenase with the same probability as to the free one. This finding indicates that the active site of dehydrogenase in its complexed form may not be blocked by aldolase.

Specific Modulation of Channelling Enzyme Complex

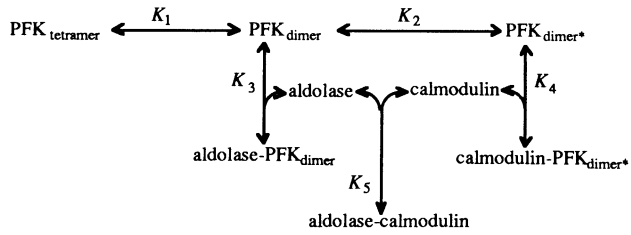
The effect of metabolites on the dynamically interacting aldolase/glycerol phosphate dehydrogenase and aldolase/glyceraldehyde 3-phosphate dehydrogenase systems have been investigated using kinetic and physico-chemical approaches. Kinetics of aldolase-catalysed conversion of fructose phosphates was analysed by coupling the aldolase reaction to the metabolically sequential enzymes, glyceraldehyde 3-phosphate dehydrogenase (unpublished work) or glycerol phosphate dehydrogenase under interacting and non-interacting conditions (unpublished work). At low enzyme concentrations polyethylene glycol was added to promote complex formation of aldolase and glycerol phosphate dehydrogenase or glyceraldehyde 3-phosphate dehydrogenase resulting in a significant increase in K_m of fructose 1, 6-bisphosphate and no change in k_{cat}. Gel-chromatographic and fluorescence measurements showed positive modulation of the interaction of aldolase with either of the dehydrogenases by fructose 1,6-bisphosphate. While the presence of fructose 1,6-bisphosphate increased the affinity of aldolase to dehydrogenases, the presence of neither fructose 1-phosphate nor dihydroxyacetone phosphate affected the dissociation constant of the heterologous enzyme complexes. We have concluded that the site for the binding of C-6 phosphate group of the substrate on aldolase is likely to be involved directly or indirectly in the interactions. Since there are several similarities in the interaction of aldolase with glyceraldehyde 3-phosphate dehydrogenase and glycerol phosphate dehydrogenase we suggested that their binding involves similar mechanism and their binding sites on aldolase might overlap. Therefore, we suggested that fructose bisphosphate modulates the formation of channelling complex

between aldolase and dehydrogenases. Obviously, the effect of metabolite levels and the alternative enzyme assemblies may mutually act upon each other. Therefore, any effect which influences the specificity of enzyme interactions may result in an alteration of substrate level, consequently, the flux of metabolic pathways (Ovádi, 1988).

Calmodulin as a Fine Modulator of Dynamic Enzyme Associations

Since phosphofructokinase and aldolase have been recognized as new target enzymes for calmodulin (Mayr, 1984; Orosz *et al.*, 1988*ab*) and calmodulin affects their catalytic properties at the concentration which is expected to exists in muscle cell (Mayr, 1984; Orosz *et al.*, 1988*a*), calmodulin may be expected to act as modulator protein on dynamic enzyme associations.

Macromolecular interactions of phosphofructokinase with functionally related enzymes seem to be important at physiological concentrations where this enzyme has sensitivity to allosteric effectors fundamentally different from that in diluted form (Boscá *et al.*, 1983). We presented evidence for the interaction of phosphofructokinase and fructose bisphosphatase at physiological concentrations with a concomitant activation of phosphofructokinase and inhibition of fructose bisphosphatase (Ovádi *et al.*, 1986). This type of interaction may be identified as an interaction inducing conformational changes (Table 1, line 2). Another metabolically related enzyme, aldolase, reduces the rate and extent of inactivation of kinase due to its dissociation on dilution, probably because it binds to the "nascent" dimer of the kinase keeping it in a partially active conformational state (Orosz *et al.*, 1988*a*). The effect of calmodulin on the concentration-dependent interaction of phosphofructokinase with aldolase has been analysed by means of a covalently attached fluorescent probe, gel penetration experiments and using a kinetic approach. We demonstrated that calmodulin perturbs both the homologous and the heterologous interactions in the phosphofructokinase/aldolase system, and elaborated a molecular model describing this effect (Orosz *et al.*, 1987). This model of the phosphofructokinase/aldolase/calmodulin interacting system rests upon the following observations: (i) the active tetrameric phosphofructokinase dissociates into inactive dimers followed by slow conformational changes; (ii) the binding of aldolase to the dimeric phosphofructokinase results in a partially active dimeric phosphofructokinase; (iii) calmodulin induces a shift from active tetrameric phosphofructokinase toward inactive dimers by binding preferentially to the conformationally changed dimeric kinase; (iv) calmodulin also binds to aldolase causing a significant decrease in the k_{cat} value of aldolase; (v) formation of a ternary protein complex phosphofructokinase-calmodulin-aldolase can be excluded; (vi) anti-calmodulin drugs eliminate the calmodulin-mediated inhibition of kinase. We considered all the equilibria between the enzyme pairs according to Scheme 2 and the equilibrium constants and the specific values of the enzymatic activity, gel penetration volume and anisotropy for enzyme species were determined experimentally. The values of activity, gel penetration volume and anisotropy from the three different types of experiments for the partially complexed protein systems were measured and calculated assuming the model (Scheme 2). The fact that the values of the calculated and measured parameters show good agreement suggests the validity of the proposed model, although more complex mechanisms cannot be excluded.



Scheme 2. Molecular model for interaction in the phosphofructokinase (PFK) / aldolase / calmodulin system. Relationships between the concentrations are shown below; for any parameter the measured value is the sum of the values for the individual PFK-containing species, weighted according to the concentration of that species divided by the total PFK concentration in dimer units.

$K_1 = [PFK_{dimer}]^2/[PFK_{tetramer}]$
$K_2 = [PFK_{dimer}]/[PFK_{dimer*}]$
$K_3 = [aldolase_{free}][PFK_{dimer,free}]/[PFK_{dimer,bound}]$
$K_4 = [calmodulin_{free}][PFK_{dimer*,free}]/[PFK_{dimer*,bound}]$
$K_5 = [aldolase_{free}][calmodulin_{free}]/[aldolase_{calmodulin\text{-}bound}]$

$[PFK]_{total\ in\ dimers} = 2[PFK_{tetramer}] + [PFK_{dimer,free}] + [PFK_{dimer*,free}] + [PFK_{dimer,bound}] + [PFK_{dimer*,bound}]$
$[aldolase]_{total\ in\ tetramer} = [aldolase_{free}] + [PFK_{dimer,bound}] + [aldolase_{calmodulin\text{-}bound}]$
$[calmodulin]_{total} = [calmodulin_{free}] + 2[PFK_{dimer*,bound}] + 2[aldolase_{calmodulin\text{-}bound}]$

Speculation about the Physiological Relevance of our Data

In muscle cells the physiological concentration of glycerol phosphate dehydrogenase is low in comparison to those of aldolase and glyceraldehyde 3-phosphate dehydrogenase (Srere, 1967). Consequently, only a small fraction of aldolase may be complexed by glycerol phosphate dehydrogenase which implies the modulating effect of fructose 1,6-bisphosphate on the interaction of aldolase and glyceraldehyde 3-phosphate dehydrogenase being relevant at physiological enzyme concentrations unless the the control of lipid synthesis is considered. Since glyceraldehyde 3-phosphate dehydrogenase preferentially interacts with the substrate-bound aldolase, the metabolic conversion of fructose 1,6-bisphosphate may be performed within the confines of the two-enzyme complex. This has been shown *in vitro* to be more efficient catalytically, as the unfavourable conversion of the aldehyde form of glyceraldehyde 3-phosphate into diol is avoided; the nascent aldehyde, emerging from the active site of aldolase, is immediately available for dehydrogenase without being released into the bulk solution. Therefore, the metabolic flux of fructose 1,6-bisphosphate specifically to 1,3-bisphosphoglycerate might be modulated by the very presence of the former. fructose 1,6-bisphosphate, a product of the central regulatory enzyme phosphofructokinase, is a positive effector of the second stage of glycolysis, acting, for example, *via* feed-forward (allosteric) activation of the enzyme pyruvate kinase and *via* enhancement of enzyme associations with cytoskeletal elements (Masters, 1981). Cellular conditions favour the binding of aldolase to dimeric phosphofructokinase, enhancing the catalytic efficiency of

phosphofructokinase *in vivo*, which may be quite different from those determined for tetrameric phosphofructokinase. The inactivation kinetics of phosphofructokinase is also strongly influenced by the enzyme concentration itself, and its associations with other proteins or cytoskeletal elements of the cell which in turn is controlled by fructose 1,6-bisphosphate. In response to a burst of activity of phosphofructokinase under physiological stress in muscle, fructose 1, 6-bisphosphate facilitates its own conversion towards ATP production by selectively enhancing the formation of channelling enzyme complex between aldolase and glyceraldehyde 3-phosphate dehydrogenase. Calmodulin may control the ability of phosphofructokinase to produce fructose 1,6-bisphosphate in a Ca^{2+}-dependent manner.

Acknowledgements: Most part of the work described in this paper are the results obtained with my colleagues Ferenc Orosz, Tania Christova, Péter Tompa and Bea Vértessy. A part of this work was carried out in collaboration with Prof. Constantino Salerno from the Institute of Biological Chemistry, University of Rome. I am very grateful to them. Research in the author's laboratory was supported by grant OTKA 315/87.

REFERENCES

Boscá, L., Aragón, J.J. & Sols, A. (1983) *J. Biol. Chem. 269*, 2100-2107
Chock, P.B. & Gutfreund, H (1988) *Proc. Natl. Acad. Sci. USA 85,* 8870-8874
Easterby, J.S. (1981) *Biochem. J. 199,* 155-161
Easterby, J.S. (1984) *Biochem. J. 219,* 843-847
Grazi, E. & Trombetta, G. (1980) *Eur. J. Biochem. 107,* 369-373
Hess, B. & Wurster, B. (1970) *FEBS Lett. 9*, 73-77
Keleti, T. & Ovádi, J. (1988) *Curr. Top. Cell Reg. 29,* 1-33
Keleti, T., Batke, J., Ovádi, J., Jancsik, V. & Bartha, F. (1977) *Adv. Enzyme Regul. 15,* 233-265
Kvassman, J., Petterson, G. & Ryde-Petterson, U. (1988) *Eur. J. Biochem. 172,* 427-431
Masters, C. (1981) *CRC Crit. Rev. Biochem. 11,* 105-144
Mayr, G.W. (1984) *Eur. J. Biochem. 143,* 513-520
Orosz, F., Christova, T.Y. & Ovádi, J. (1987) *Biochem. Biophys. Res. Comm. 147,* 1121-1128
Orosz, F., & Ovádi, J. (1987) *Biochim. Biophys. Acta 915,* 53-59
Orosz, F., Christova, T.Y. & Ovádi, J. (1988a) *Biochim. Biophys.Acta 957,* 293-300
Orosz, F., Christova, T.Y. & Ovádi, J. (1988b) *Molec. Pharm. 33,* 678-682
Ovádi, J. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, S., Keleti, T. & Trón, L., eds.), pp. 203-216, Akadémiai Kiadó, Budapest, and Elsevier, Amsterdam
Ovádi, J. (1988) *Trends Biochem. Sci. 13*, 486-490
Ovádi, J. & Keleti, T. (1978) *Eur. J. Biochem. 85,* 157-161
Ovádi, J., Aragón, J.J. & Sols, A. (1986) *Biochem. Biophys. Res. Comm. 135,* 852-856
Ovádi, J., Salerno, C., Keleti, T. & Fasella, P (1978) *Eur. J. Biochem. 90,* 499-503
Ovádi, J., Tompa, P., Vértessy, B., Orosz, F., Keleti, T. & Welch, G.R. (1989) *Biochem. J. 257,* 187-190
Patthy, L. & Vas, M. (1978) *Nature 276,* 274-276
Salerno, C., Ovádi, J., Keleti, T. & Fasella, P. (1982) *Eur. J. Biochem. 121,* 511-517
Srere, P.A. (1967) *Science 158,* 936-937
Srere, P.A. (1987) *Annu. Rev. Biochem. 56*, 89-124
Srivastava, D.K. & Bernhard, S.A. (1987) *Ann. Rev. Biophys. Biophys. Chem. 16,* 175-204
Srivastava, D.K. & Bernhard, S.A. (1986) *Curr. Top. Cell. Reg. 28,* 1-68
Tompa, P., Batke, J. & Ovádi, J. (1987) *FEBS Lett. 214,* 244-248
Vértessy, B. & Ovádi, J. (1987) *Eur J.Biochem 164,* 655-659
Welch, G.R. (ed.) (1985) *Organized Multienzyme Systems: Catalytic Properties,* Academic Press, New York

Chapter 23

Temporal Analysis of the Transition between Steady States

JOHN S. EASTERBY

MOST STUDIES of metabolic control have concentrated on defining pathway flux and its determinants. In particular the fine-control of enzyme activity and the homeostatic maintenance of steady states has been a focus of attention. In the cell many changes are more dramatic and involve large changes of flux. Much less attention has been given to the equally important topic of the time-scale on which such changes, and metabolic processes generally, operate. A full description of regulation of a system must include such temporal analysis. It is insufficient to ask of a pathway, cell or organism "How fast does it go?", it is also necessary to ask "How long does it take to get there?". This temporal responsiveness sets the time-scale on which metabolism operates and is best described by the pathway transition or transient time, τ. The reluctance to study pathway dynamics probably arises partly from a mistaken belief that real-time measurements or a detailed knowledge of pathway kinetics is necessary. Either way the experimentation would be difficult.

The study of pathway transients developed, almost by accident, out of a related interest in the optimization of coupled or consecutive enzyme reactions. This area of investigation was initiated by McClure (1969) with a description of the kinetic behaviour of a two-enzyme system in which the first enzyme was severely rate-limiting and the single intermediate was converted to a measurable product by a single coupling enzyme. The two-enzyme system was later extended to three (Barwell & Hess, 1970) and finally developed to describe a system containing an unlimited number of coupling enzymes (Easterby, 1973). In all of these studies the rate-limiting nature of the first enzyme of the series ensured that the intermediate concentrations in the sequence were low and the coupling enzymes followed pseudo-first-order kinetics with respect to the intermediates. In all cases the steady-state production of end-product by the system was preceded by a lag period during which the intermediates of the sequence were accumulating to their steady-state levels. If the steady-state asymptote to the progress curve in product formation was projected to the time axis, it intercepted at the lag or transient time. In the case of a two-enzyme system, at least 4.6

John S. Easterby ● Department of Biochemistry, The University, P. O. Box 147, Liverpool, L69 3BX, U.K.

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

transient times must elapse before the intermediate concentration and the rate of product formation approach their steady-state values to within 1% (Fig. 1 of Easterby, 1973). For progressively longer sequences this time is proportionately decreased until in the case of very long sequences the system obeys a step function and enters the steady state immediately on reaching the transient time (Easterby, 1984).

This simple system was never intended to represent the behaviour of metabolic pathways, but it did indicate some principles which might apply more generally to pathways. Firstly the transient time for the sequence was found, rather surprisingly, to be independent of the initial, rate-limiting enzyme. Secondly associated with each secondary or coupling enzyme in the sequence was a transient time corresponding to the reciprocal of the pseudo-first-order rate constant for the reaction catalysed and therefore corresponding also to the mean lifetime or turnover time of the intermediate. Thirdly, these transient times were additive and the total transient was their sum. Fourthly the intercept on the ordinate axis (Fig. 1) corresponded to the negative sum of the steady-state intermediate concentrations of the sequence.

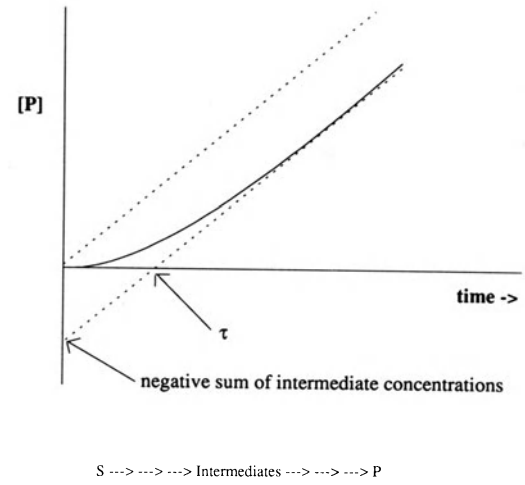


Figure 1. Relationship between product concentration and time in a coupled enzyme system. Substrate S is converted to end-product P through several intermediates. The initial enzyme of the sequence is assumed to determine the flux. The steady-state asymptote to the progress curve intersects the time axis at the transient or transition time, τ. The ordinate axis is intersected at the negative sum of the intermediate concentrations. The line drawn parallel to the steady-state asymptote and passing through the origin represents the hypothetical time-course if no intermediates were involved. The displacement of this line from the asymptote, along the product axis, is equal to the sum of steady-state intermediate concentrations, irrespective of the kinetic mechanisms of the enzymes involved.

The transient times associated with individual enzymes in this sequence were given by the K_m/V ratios. This suggested that not only enzymes with low activity but also those with low affinity for their substrates could generate appreciable transient times and the term "time-limiting enzyme" was coined to describe them (Easterby, 1973; Heinrich & Rapoport, 1975). If such an enzyme were to follow a branch point in a pathway, then it could determine the distribution of flux between branches as it would only enter its steady state if the feeder pathway were sustained for a sufficient length of time. Almost immediately such an enzyme was discovered in the *arom* complex of *Neurospora*. This was shikimate kinase and was a member of what was thought to be a multienzyme complex but is now known to be a multifunctional protein (Welch & Gaertner, 1976). The conclusions drawn from the

model system were subsequently applied to several other pathways and multienzyme complexes, but always with a certain danger in view of the very restricted original model.

A Generalization of the Description of the Transient

It soon became clear that the coupled enzyme model was a special case of a much more general approach to pathway transience. It can be seen from Fig. 1 that, had there been no intermediates present in the pathway, then the progress curve for product formation would have followed a straight line with slope equal to the steady-state rate and passing through the origin. The difference between this line and the actual progress curve in product formation is clearly equal to the concentration of accumulated intermediates, irrespective of the kinetic description of the model. Thus the transient time can be deduced from a consideration of mass conservation without the need to model the system. The outcome is that associated with each intermediate is a transient given by:

$$\tau = [\mathrm{I}]_{ss}/J \tag{1}$$

where $[I]_{ss}$ represents the steady-state concentration of intermediate and J the steady-state flux. As before the overall transient is a simple sum of the individual intermediate transients. Thus the same basic interpretation of transience which was applied to the earlier pseudo-first-order model was shown to obtain and indicated the value of even a simple kinetic model in the development of a general approach. This general theory was first stated by Easterby (1981) but had also been recognized in a more limited form by Bartha & Keleti (1979). The difference between this description of the transient and that for the coupled reaction model was that it was now seen generally that the transient was associated with intermediates, not enzymes. The transient time was the time required to generate the pools of intermediates. It was also associated with individual enzymes in the simple model as the reactions considered were irreversible. Thus transient times are generally associated with intermediates. They are additive in determining the overall pathway transient. They are not determined by a single enzyme but are dependent on all enzymes in the pathway up to the next irreversible step (Easterby, 1981).

This may be summarized as follows:

$$\tau_i = [\mathrm{I}_i]_{ss}/J \tag{2}$$

$$\tau = \sum_{i=1}^{n} ([\mathrm{I}_i]_{ss}/J) = \sum_{i=1}^{n} \tau_i \tag{3}$$

where I_i signifies the ith of n intermediates in the pathway.

This approach to the analysis of pathway transient times has the striking advantage for the experimentalist of being independent of any prior assumption of a kinetic model to describe the pathway. Simple measurement of intermediate concentrations and fluxes serves

to identify the time scale on which the pathway operates and the contributions of individual steps to that time-scale.

So far no comment has been made as to what constitutes a pathway. For the purpose of this analysis the pathway may be rather broadly defined. The flux across the initial boundary must be constant with time and the final boundary may be placed anywhere. The end-product constitutes the sum of all material crossing the final boundary. In measuring the transient time it is assumed that initially no intermediate is present and the flux is zero, thus the transient time is associated with the establishment of the pathway from rest. Of course this is an unrealistic view of what happens in the cell where passage between steady states rather than their establishment from rest is the more common occurrence. However, such a situation can be accommodated very easily. It turns out that the transient times are functions of state and are independent of the route by which the steady state was reached. It is therefore easy to show that the time required for a transition between steady states can be described in terms of the transient times associated with the establishment of each steady state from rest. Thus the transient time for a transition from steady state A to steady state B is given by:

$$\tau = \tau_B - \frac{J_A}{J_B}\tau_A \tag{4}$$

where τ_A and τ_B represent the transients associated with the establishment of steady states A and B from rest and J_A and J_B are their respective fluxes.

Variation in the Rate of Input to the Pathway

The description of the pathway so far given assumes that the initial reaction or boundary involves a constant rate of input. In general this will mean that the initial step is flux generating. This restriction may be lifted if allowance is made for variation in the rate of input. This generates an additional transient time given by the definite integral:

$$\tau = \frac{1}{v_{ss}}\int_0^{v_{ss}} t\,dv \tag{5}$$

where v is the rate of input and v_{ss} represents the steady-state input rate (ie. the steady-state flux). This term therefore allows for variation of the rate of input (Easterby, 1986). This variation arises either from substrate depletion or feedback on the initial boundary of the system. Substrate depletion is easily overcome experimentally but feedback is more of a problem. However, in practice the magnitude of this transient is likely to be small compared to the general pathway transient. Where it is necessary to consider it, the only answer may be to resort to computer simulation (Easterby, 1986). In the case of negative feedback the transient will be negative and will reduce the overall transient time. One function of negative feedback may be to accelerate the attainment of the steady state in this way. Apparent over capacity of some enzymes may be necessary in order to ensure a rapid transition between

steady states with the final flux being determined by feedback. If both substrate depletion and feedback on the initial boundary are absent then this transient reduces to the characteristic time of the initial enzyme and again will usually be correspondingly small and negligible.

Kinetic Models for the Determination of Transient Times

The discussion so far has centred on how transient times are measured experimentally, namely from fluxes and pool sizes and the theory does not constitute a model of pathway behaviour. It is often useful to have a kinetic model of how the steady-state pool sizes and fluxes are determined. Given such models it is possible to compare theory with experiment. A model in this context merely means some function which will adequately represent the transformation/removal of an intermediate within the pathway during the steady state. The approach is therefore almost phenomenological in the sense that the function does not require great generality but must merely represent the enzyme's rate adequately under the restricted steady-state conditions being considered. Functions are of the following form:

$$\frac{d[I]}{dt} = v_{ss} - f([I], x, y, z\cdots) = 0 \tag{6}$$

where x, y, z ... etc are any modulators of enzyme activity. These functions are generally fairly simple where irreversible conversion of the intermediate is involved. They become complex where reversible processes are involved (Easterby, 1981, 1985). Some common cases are as follows:

(*a*) *Conversion of intermediate follows pseudo-first-order kinetics:*

$$\tau = K_m/V \tag{7}$$

(*b*) *Conversion follows Michaelis-Menten kinetics:*

$$\tau = K_m/(V - J) \tag{8}$$

A closer examination of systems of this sort shows that if enzyme capacity exceeds flux by a factor of more than two, then first order behaviour gives a reasonable representation of transient response, even for enzymes obeying Michaelis-Menten kinetics (Easterby, 1981).

(*c*) *Conversion follows the Hill equation:*

$$\tau = \frac{S_{0.5}}{J}\left(\frac{V}{J} - 1\right)^{-\frac{1}{h}} \tag{9}$$

Here h represents the Hill coefficient and $S_{0.5}$ the substrate concentration for half-maximal rate. In this case, if the enzyme is highly cooperative, then τ is determined by $S_{0.5}$ and flux and is independent of the enzyme's capacity.

In all of these analyses it has been assumed that the transient time is due to the accumulation of free intermediate pools within the pathway. In practice enzyme-bound intermediates might also contribute to the transient. In this case τ for a Michaelis-Menten enzyme is given by:

$$\tau = \frac{[E]_t}{V} + \frac{K_m}{V - J} = \frac{1}{k_{cat}} + \frac{K_m}{V - J} \tag{10}$$

where $[E]_t$ represents the total enzyme concentration and k_{cat} its catalytic constant; the first term in these expressions represents the contribution of enzyme-bound intermediate. The conclusion is that the contribution of enzyme-bound intermediate to the transient is only significant when enzyme concentration approaches K_m; in other words it will generally be insignificant. A complete description of the transient would, of course, have to take into account variation in the rate of input. The complete description is therefore as follows:

$$\tau = \frac{1}{v_{ss}} \int_0^{v_{ss}} t\,dv + \sum_{i=1}^{n} \left(\frac{1}{k_{cat,i}} + \frac{K_{m,i}}{V_i - v_{ss}} \right) \tag{11}$$

the first term representing the variation in flux, the second the mean lifetimes of enzyme-substrate complexes and the third the lifetimes of the pools of free intermediates.

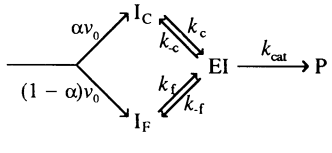
One use of modelling of this sort is to identify diffusional restrictions or substrate channelling within experimental systems. The transients so far described have been derived on the assumption that both enzymes and substrates are present in a system where free diffusion can occur. If measured transients correspond to theoretical values then one may assume that this is a valid assumption. If transients are greater than those expected on the basis of theory then some form of diffusional restriction, owing perhaps to high intracellular viscosity, may be occurring. Conversely if the transient times are smaller than expected substrate or intermediate channelling is indicated.

The Analysis of Substrate Channelling

In recent times much attention has been directed to substrate channelling within multi-enzyme complexes and multifunctional proteins. By this process, intermediates in the reaction chain are thought to be transferred directly between enzymes of the complex without the need for diffusion into the bulk medium. Thus it might be envisaged that at least three pools of intermediate exist within the pathway. One of these would occupy the bulk medium, a second would correspond to enzyme-bound intermediate and the third to intermediate trapped or channelled within the complex but not directly associated with enzyme active sites. The system described is thus a "leaky" one in that not all intermediate is retained within the complex, some is allowed to enter the bulk medium and must diffuse back to the complex if it is to be converted through to product. Such a system has three possible advantages. Firstly

the existence of the multienzyme complex may serve to isolate the reactions and intermediates of a pathway. Secondly coordinated regulation of enzymes within the complex may be possible (Welch & Gaertner, 1976; Nicholson *et al.*, 1987). Thirdly a minimization of the transient time for the pathway may occur thus obviating the need for the accumulation of intermediates. The concentration of intermediate seen by enzymes of the complex is greater than if free diffusion were allowed and therefore, according to the mass conservation principle, less material and time is wasted in generating unwanted intermediate pools. Channelling offers no flux advantage as flux is still determined by the initial rate of input to the pathway.

It is clear that channelling can be analysed in terms of the transient times involved. Channelling leads to a reduction in the sizes of intermediate pools and therefore to the associated transient times. At an empirical level the advantage gained from channelling can be assessed by comparing the measured transient for the complex with that derived from a consideration of free solution kinetics as detailed previously. However, in order to gain a better insight into the mechanism, advantages and potential need or usefulness of channelling, a model is required. Fig. 2 contains what may be regarded as the minimum model consistent with a realistic view of the channelling process. Here channelling of intermediate I to a single enzyme E within a multienzyme complex or multifunctional protein is considered. To keep the model relatively simple it is assumed that the release of the intermediate from the preceding enzyme is irreversible with proportion α being channelled and 1 - α being released into free solution. Thus the channel is "leaky" and α represents the efficiency of channelling. α will be in the range 0 to 1. It is assumed that no direct exchange of any significant extent occurs between channelled and free intermediate as this would essentially abolish the channel. However, some exchange must be contemplated through the enzyme complex, EI, if the intermediate released into the bulk medium is ever to return to the pathway and be converted to end-product. Thus we must allow both ends of the channel to be somewhat "leaky". As usual the transient time for this system is given simply by the sum of enzyme-bound and free intermediate concentrations divided by the steady-state flux and can be determined without recourse to the solution of differential equations. The descriptions of the transient times derived for the scheme are given in Fig. 2.



$$\tau = (1 - \alpha)\tau_F + \alpha\tau_C, \text{ where}$$

$$\tau_F = \frac{1}{k_{cat}} + \frac{K_{mF} + K_{DC}}{V - v_0} = \frac{1}{k_{cat}} + \frac{K_{mF} + K_{DF}/\beta}{V - v_0}$$

$$\tau_C = \frac{1}{k_{cat}} + \frac{K_{mC} + K_{DF}}{V - v_0} = \frac{1}{k_{cat}} + \frac{K_{mF}/\beta + K_{DF}}{V - v_0}$$

Figure 2. A model of substrate channelling within multienzyme complexes and multifunctional proteins. I_C represents channelled intermediate, I_F intermediate released into free solution and v_0 represents the steady-state flux. τ_F and τ_C are the transient times associated with the routes involving complete release and complete channelling of intermediate respectively (ie. $\alpha = 0$ and $\alpha = 1$). K_{mF} and K_{mC} are the Michaelis constants of intermediate associated with these routes and K_{DF} and K_{DC} the corresponding dissociation constants of intermediate from the enzyme into free solution and into the interior of the enzyme complex respectively. The *channelling efficiency* α represents the fraction of intermediate channelled; the *channelling advantage* β the factor by which K_m and K_D are reduced within the complex and V the maximum velocity of enzyme E.

There are two ways of defining the efficiency. This may be based either on the distribution of flux, α, or on the distribution of intermediate between the complex and free solution. These are not the same and it is clear that the advantage in reduction of intermediate pool size comes from the distribution of intermediate and is related to the transient time. The distribution of flux is important in so far as it contributes towards the determination of pool size.

From the equations of Fig. 2 it can be seen that the total transient time is a linear combination of the transient times associated with the two extreme routes. Namely total channeling ($\alpha = 1$) and total release of intermediate into free solution ($\alpha = 0$). Each transient is weighted by the flux through the corresponding route. The same result was found by Ovádi *et al.* (1989) but here a more complex model is considered and the contributing transient times are correspondingly more complicated. The transient associated with the channelling route, τ_C, comprises terms attributable to enzyme-substrate complex, channelled intermediate and intermediate released from the enzyme-substrate complex into the bulk medium. It will be noticed that even with complete channelling, intermediate may still enter the bulk medium by this route and its concentration will be determined by the dissociation constant of the enzyme-substrate complex. The transient for the completely unchannelled route, τ_F, is analogous to τ_C but now intermediate may be released into the "interior" of the complex by dissociation.

The single most important kinetic consequence of channelling is the increased concentration of intermediate seen by the enzyme. This will manifest itself in a reduction in transient time and an apparent reduction in K_m. The reduction in K_m will result principally from an increase in the *on* constant for the formation of EI complex, owing to the increased probability of an encounter between enzyme and intermediate. The dissociation constant of the complex will be similarly reduced. In Fig. 2, the factor by which K_m is reduced in the complex compared to free solution has been called β, the channelling advantage. Assuming that the improved access to intermediate is the main consequence of complex formation and other aspects of kinetic behaviour are unaltered then the dissociation constant will be reduced by the same factor. Thus the description of the transients can be simplified and expressed in terms of the free solution K_m and dissociation constant (Fig. 2).

Two quantities now describe the channelling process. The channelling efficiency, representing the division of flux, and the channelling advantage, representing the increased accessibility or binding of intermediate to the enzyme. Analysis of the equations of Fig. 2 leads to the following conclusions:

1. There is a lower limiting value to the transient time. This is reached at high protein concentration and is equal to $1/k_{cat}$, the mean lifetime of intermediate in the EI complex. Therefore, even with 100% channelling and high channelling advantage, no kinetic advantage is received from channelling at high protein concentration as the transient time is the same as for the free solution case. By high protein concentration we mean concentrations comparable to K_m.

2. For τ_C and τ_F to be distinguishable and a reduction in transient time to be seen, K_{mF}

must be much greater than K_{DF}. This equivalent to saying kcat must be much greater than the *off* constant of the EI complex ($k_{cat} >> k_{-f}$). Thus enzymes exhibiting rapid-equilibrium kinetic mechanisms in free solution make poor candidates for channelling.

Returning to Fig. 2, while the channelling efficiency will be in the range 0-1, channelling advantage will generally be greater than unity. As it approaches infinity we have the situation in which there is no pool of intermediate internal to the complex but the intermediate is transferred directly between enzyme active centres.

The channelling efficiency in this system is given by:

$$\alpha = \frac{K_{mF} + K_{DF}/\beta - (\tau - 1/k_{cat})(V - v_0)}{(K_{mF} - K_{DF})(1 - 1/\beta)} \tag{12}$$

Various simplifications to this expression are possible if channelling is efficient. That is to say if β is large or $K_{mF} >> K_{DF}$. It should then be possible to evaluate the transients for the channelled and un-channelled routes. Alternatively, if α can be assessed by isotopic means, β becomes accessible. In the simplest case where β is large and K_{mF} is much greater than K_{DF}, α is given by:

$$\alpha = \frac{1 - (\tau - 1/k_{cat})(V - v_0)}{K_{mF}} \tag{13}$$

In the foregoing account I hope to have shown that a simple study of intermediate concentrations and fluxes can provide considerable information on the time domain and dynamics of metabolic processes. Such quantities should be readily accessible to measurement by the experimentalist.

Unification of Flux and Temporal Control Theories

Temporal control and flux control within pathways deal in the same quantities, namely pool sizes and fluxes. It may be readily shown that these control processes are related through the control coefficients. A temporal control coefficient may be defined such that:

$$C^{\tau}_{[E]_i} = C^{\Sigma S}_{[E]_i} - C^{J}_{[E]_i} \tag{14}$$

where ΣS represents the sum of intermediate pools and J represents the flux.

A summation property also applies to the temporal control coefficients:

$$\sum_{i=1}^{n} C^{\tau}_{[E]_i} = -1 \tag{15}$$

This reflects the fact that the transient decreases as the enzyme concentrations within the pathway increase. This topic is developed more fully by Acerenza in Chapter 25 of this book.

REFERENCES

Bartha, F. & Keleti, T. (1979) *Oxid. Commun. 1,* 75-84
Barwell, C. J. & Hess, B. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351,* 1531-1536
Easterby, J. S. (1973) *Biochim. Biophys. Acta 293,* 552-558
Easterby, J. S. (1981) *Biochem. J. 199,* 155-161
Easterby, J. S., (1984) *Biochem. J. 219,* 843-847
Easterby, J. S. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, S., Keleti, T. & Trón, L., eds.), pp. 145-158, Akadémiai Kiadó, Budapest
Easterby, J. S. (1986) *Biochem. J. 233,* 871-875
Heinrich, R. & Rapoport, T. A. (1975) *BioSystems 7,* 130-136
McClure,W.R. (1969) *Biochemistry 8,* 2782-2786
Nicholson, S., Easterby, J. S. & Powls, R. (1987) *Eur. J. Biochem. 162,* 423-431
Ovádi, J., Tompa, P., Vértessy, B., Orosz, F., Keleti, T. & Welch, G. R. (1989) *Biochem. J. 257,* 187-190
Welch, G. R & Gaertner, F. (1976) *Arch. Biochem. Biophys. 172,* 476-489

Chapter 24

Sensing of Chemical Signals by Enzymes

JACQUES RICARD, JEAN BUC, NICOLAS KELLERSHOHN
and JEAN-MICHEL SOULIÉ

LIVING SYSTEMS are able to sense the intensity of chemical signals originating from the external milieu and are able to detect whether this intensity increases or decreases. Chemotaxis of bacteria represents a striking example of these sensory properties defined at a rather simple level (Koshland, 1979, 1980*ab*, 1981). Bound enzyme systems in which diffusion is a limiting process may also display these sensory properties (Engasser & Horvath, 1974, 1976; Ricard, 1987). This implies that the system possesses a memory and that its response is different depending on whether the concentration of a ligand increases or decreases. In other words the response of the system is sensitive to its history. Nucleic acids may display metastable secondary structures upon their titration and therefore may exhibit hysteresis effects (Revzin *et al.*, 1973, Neumann, 1973, Schneider, 1976).

The aim of this chapter is to show that isolated enzymes in free solution may exhibit apparent multiple steady states and behave as true biosensors. A more detailed account of these ideas has been published elsewhere (Ricard *et al.*, 1988*ab*, Soulié *et al.*, 1988).

Sensing Chemical Signals and the Existence of Meta-Steady States

If one neglects product inhibition, and if there is a large excess of a substrate over the enzyme concentration, the equation that describes the progress curve of product appearance assumes the following form:

$$\frac{[P]}{[E]_t} = \alpha + \frac{v_{ss}t}{[E]_t} + \sum_{i=1}^{n} \psi_i e^{-\lambda_i t} \tag{1}$$

where [P] is the product concentration at time t, $[E]_t$ the total enzyme concentration, v_{ss} the steady-state rate, ψ_i and λ_i collections of rate constants and ligand concentrations equivalent to relaxation amplitudes and time constants; α is a collection of rate constants and ligand concentrations, and may take positive or negative values depending on whether the reaction displays a burst or a lag. The numerical values of v_{ss} and λ_i do not depend on

Jacques Ricard, Jean Buc, Nicolas Kellershohn and Jean-Michel Soulié ● Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

whether the concentration of a ligand is decreased or increased, but those of α and ψ_i do. This means that the numerical values of these parameters depend on the history of the system.

Classical enzyme kinetics implicitly postulates that as the product $v_{ss}t/[E]_t$ becomes significant the exponential terms of eqn. (1) may be neglected. This means that the transient phase is well separated in time from the steady-state phase. But if this is not so and if, for instance, the nth transient is extremely slow, one has the following approximate relationship:

$$e^{-\lambda_n t} \approx 1 - \lambda_n t \tag{2}$$

Eqn. (1) may thus be rewritten as follows:

$$\frac{[P]}{[E]_t} = \alpha + \psi_n + \left(\frac{v_{ss}}{[E]_t} - \lambda_n \psi_n\right) t + \sum_{i=1}^{n-1} \psi_i e^{-\lambda_i t} \tag{3}$$

and the steady state that can be measured is only apparent. For reasons that will appear later, it is called the *meta-steady state*. The corresponding meta-steady-state rate v^*_{ss} is expressed as follows:

$$v^*_{ss} = v_{ss} - \lambda_n \psi_n [E]_t \tag{4}$$

From this expression, it becomes obvious that the meta-steady-state rate depends on the history of the system, as ψ_n depends on this history.

An enzyme mechanistic device that may generate this type of behaviour is the occurrence of a "slow" conformational transition of the enzyme. In this context "slow" means slow by comparison with the other reaction steps. The simplest model that displays this type of conformational transition is the *mnemonical model* (Ricard *et al.*, 1974). In this model it is postulated that the enzyme retains, or recalls, for a while the conformation stabilized by the last product of the reaction sequence before "slowly" relapsing to the other conformation. A number of enzymes have been shown to display this type of behaviour (Neet & Ainslie 1980; Cornish-Bowden & Cárdenas 1987). For a one-substrate one-product monomeric enzyme, this situation is depicted in Fig. 1.

Thus the free enzyme exists under two metastable states and this may generate the type of behaviour predicted by eqns. (3-4). The evolution with time of the ES complex for such a simple system is shown in Fig. 2, which shows the existence of two meta-steady states of this complex that are different from the real steady state. These meta-steady states rely on the existence of metastable states of the enzyme.

Meta-steady-state rates display two unique properties: first, even with a simple one-

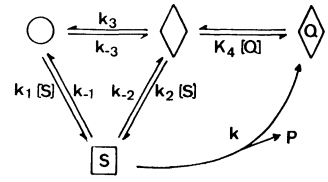


Figure 1. The simple mnemonical model used to simulate hysteresis of the meta-steady state. S is the substrate, P a product, and Q the product that stabilizes the rhombus conformation.

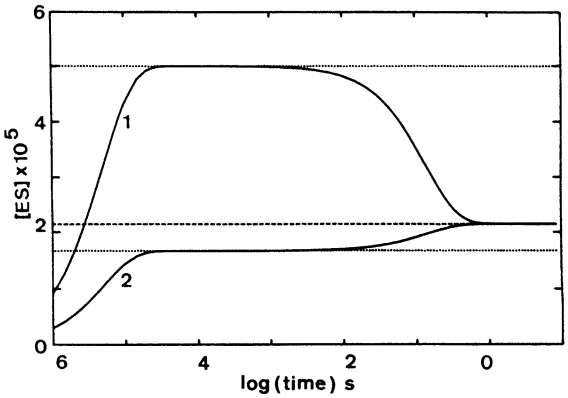


Figure 2. The existence of meta-steady state during the approach to the steady state. The model simulated is that of Fig. 1. The two progress curves 1 and 2 show the existence of a meta-steady state of the enzyme-substrate complex before the real steady state is reached. The numerical values of the rate constants, used for simulation are as follows: $k_1 = 10$, $k_{-1} = 10^5$, $k_2 = 2$, $k_{-2} = 1$, $k_3 = 1$, $k_{-3} = 5 \times 10^{-5}$. The product Q is assumed to be absent in a pre-incubation mixture for curve 1 and to be present for curve 2 at a concentration such that $K_4[Q]$ equals 10^5. The product is then assumed to be chased away immediately before the reaction is started. No product is thus present at time zero of the reaction, but this reaction is initiated with different concentrations of the two free enzyme forms for curves 1 and 2. The steady-state concentration of ES corresponds to the dashed line and the two meta-steady states to the dotted lines.

substrate one-product monomeric enzyme the product may behave as an activator; second, the meta-steady-state rate assumes different values for the same concentration of the product depending on whether this product concentration is reached after an increase or after a decrease of concentration. These properties are illustrated by computer in Fig. 3.

If the real steady-state rate were measured, neither of these two surprising properties would be found. The existence of two different meta-steady-state rates for the same ligand concentration is due to two initial positions of the enzyme equilibrium, depending on whether the actual concentration of the product is reached after a decrease or an increase of an initial product concentration.

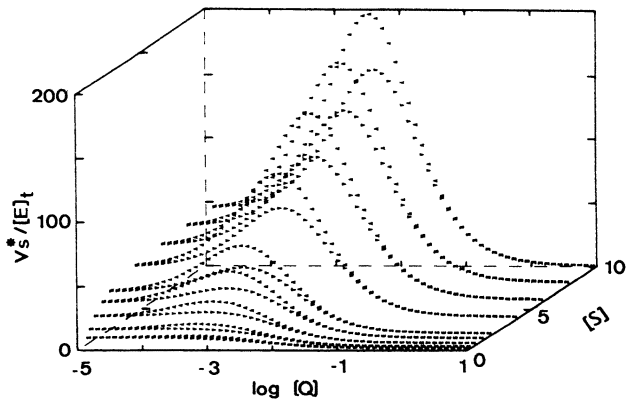


Figure 3. Hysteresis of the meta-steady-state rate of the reaction. The values of the rate and equilibrium constants are: $k_1 = 1$, $k_{-1} = 10^{-3}$, $k_2 = 10^4$, $k_{-2} = 5 \times 10^{-2}$, $k_3 = 20$, $k_{-3} = 1$, $k = 10^3$, $K_4 = 4 \times 10^3$. The arrow-heads indicate the direction of the concentration change.

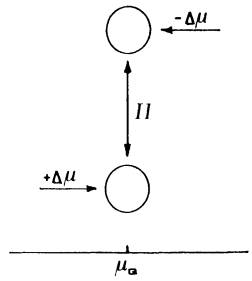


Figure 4. The sensing potential Π. At constant final chemical potential of the ligand Q a positive or a negative force Δμ generates a difference of potential of an enzyme species (the circle in this figure).

The Sensing Potential

The existence of hysteresis (that is the existence of two different values of the meta-steady-state rate for the same product concentration) of the meta-steady-state rate implies that the various enzyme species that appear during the reaction sequence should also display hysteresis. Expressed in more rigorous thermodynamic terms, this means that applying a positive or a negative force, Δμ, at constant chemical potential of the ligand, should result in two different values of the chemical potential of the same enzyme species. This is depicted in Fig. 4. The difference of chemical potential of the same enzyme species, depending on whether the force applied is positive or negative, may be called the *sensing potential* Π and defined as follows:

$$\Pi = \mu_f - \mu_r \tag{5}$$

where μ_f and μ_r are the chemical potentials of an enzyme species obtained upon applying a positive and a negative force, respectively. A positive or a negative value of this potential means that the system is able to sense the direction of a change of a chemical signal. The value of the potential Π expresses the ability of the system to act as a biosensor.

The sensing potential of an enzyme species is a function of both the force (positive or negative) and of the chemical potential of the ligand. Of particular interest is the dependence of this potential with respect to the potential of the ligand Q. As a rule , the sensing potential of any of the enzyme species depends on the potential of Q. This means that for certain concentrations of Q the system is unable to sense the direction of a concentration change, whereas for other concentrations the sensitivity of the system to the direction of the concentration change is maximum. An example of the variation of the sensing potential of one of the free enzyme form (the "circle" form) as a function of the potential of Q is shown in Fig. 5.

Hysteresis of Chloroplast Fructose 1,6-Bisphosphatase

Chloroplast fructose 1,6-bisphosphatase is a tetramer made up of apparently identical subunits. It plays a key role in the regulation of the Benson-Calvin cycle. In its oxidized state at pH 7.5 it is nearly totally devoid of activity. It may, however, regain full activity if it is incubated with low concentrations of fructose 2,6-bisphosphate.

If the inactive oxidized enzyme is incubated with a reaction mixture containing the

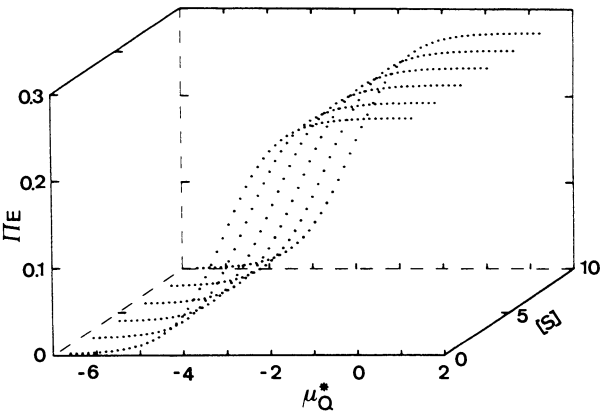


Figure 5. Variation of the sensing potential of an enzyme species as a function of the reduced chemical potential of the ligand Q. The values of the rate and equilibrium constants (in arbitrary units) used for simulation are those given in the legend of Fig. 3. μ^*_Q is the reduced chemical potential ($\mu_Q - \mu^\circ_Q$) of the product Q.

substrate (fructose 1,6-bisphosphate) and the analogue (fructose 2,6-bisphosphate) it develops a quite significant activity after a slow lag. In a second experiment the enzyme is preincubated with fructose 2,6-bisphosphate in the presence of magnesium, and then these ligands are chased away by dilution, the enzyme is mixed with the same reaction medium as in the previous experiment. Under these conditions the reaction displays no lag (Fig. 6). The enzyme thus retains the conformation stabilized by fructose 2,6-bisphosphate after chasing this ligand away, and this conformation is the active one.

Since the enzyme may retain the conformation stabilized by fructose 2,6-bisphosphate, the meta-steady state of this reaction should display a hysteresis loop. This may be shown by incubating oxidized inactive fructose bisphosphatase with fructose 2,6-bisphosphate and magnesium. These ligands are chased away and the apparent steady-state rate (the meta-steady-state rate) is measured in the presence of the concentration of fructose 2,6-bisphosphate immediately higher or lower than the concentration used for the pre-incubation. One may observe that the meta-steady-state rate of the reaction is different depending on the enzyme has been pre-incubated at a higher or a lower concentration of fructose 2,6-bisphosphate. This hysteresis cycle is shown in Fig. 7.

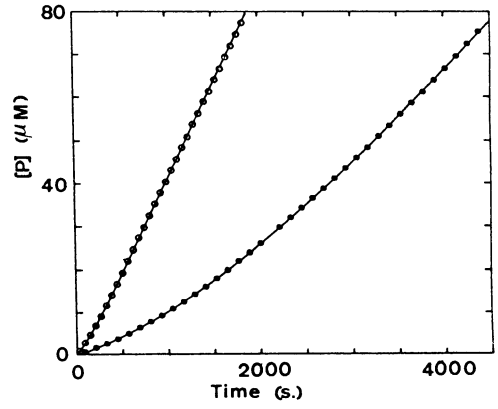


Figure 6. Memory effects of fructose bisphosphatase. The progress curve of reaction is monitored in the presence of 0.37 mM fructose 2,6-bisphosphate and 2.7 mM magnesium. In the case of the upper curve (o), the enzyme has been pre-incubated (prior to the assay) for 6 hours with 2.7 mM magnesium and 1 mM fructose 2,6-bisphosphate. These ligands are then chased away by dilution. In the case of the lower curve (●), the enzyme has been incubated for the same time with 2.7 mM magnesium, in the absence of fructose 2,6-bisphosphate which is then chased away by dilution before the assay. The reaction mixture is thus the same for both assays.

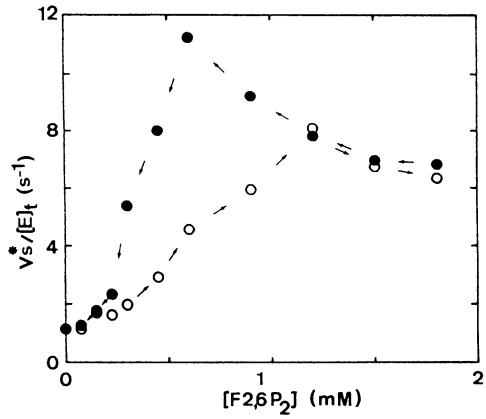


Figure 7. Hysteresis loop of meta-steady-state rate of fructose bisphosphatase reaction as a function of fructose 2,6-bisphosphate concentration. The enzyme is incubated with 2.7 mM magnesium and various concentrations of fructose 2,6-bisphosphate before it is assayed in the presence of an immediately inferior (o) or superior (●) concentration of fructose 2,6-bisphosphate. The meta-steady-state rate is estimated over the first 15 min of the reaction.

Concluding remarks

The result that some conformational transitions of enzymes may result in a hysteresis loop of the apparent steady-state velocity, opens new vistas on the molecular mechanisms of enzyme regulation. It implies that a single enzyme in free solution may sense the *direction* of variation of a chemical signal and not only its *intensity*.

Chloroplast fructose bisphosphatase certainly plays a major role in chloroplast carbon metabolism and displays this kind of dynamic behaviour. It is tempting to speculate that this situation is not unique in metabolic control.

REFERENCES

Cornish-Bowden, A. & Cárdenas, M. L. (1987) *J. Theor. Biol. 124,* 1-23
Engasser, J. M. & Horvath, C. (1974) *Biochemistry 13,* 3855-3859
Engasser, J. M. & Horvath, C. (1976) in *Applied Biochemistry and Bioengineering* (Wingard, L. B., Katchalski-Katzir, E. & Goldstein, L. eds.), Vol. 1, pp. 127-220, Academic Press, New York
Koshland, D. E., Jr. (1979) *Physiol. Res. 59,* 812-862
Koshland, D. E., Jr. (1980) *Bacterial Chemotaxis as a Model Behavioral System,* Raven Press, New York
Koshland, D. E., Jr. (1980) *Annu. Rev. Neurosci. 3,* 43-75
Koshland, D. E., Jr. (1981) *Annu. Rev. Biochem. 50,* 765-782
Neet, K. E. & Ainslie, G. R., Jr. (1980) *Methods Enzymol. 64,* 192-226
Neumann, E. (1973) *Angew. Chem. Internat. Edit. 12, 356-369*
Revzin, A., Neumann, E. & Katchalski, A. (1973) *J. Mol. Biol. 79,* 95-114
Ricard, J., Meunier, J.-C. & Buc, J. (1974) *Eur. J. Biochem. 49,* 195-208
Ricard, J. (1987) *J. Theor. Biol. 128,* 253-278
Ricard, J. & Buc, J. (1988) *Eur. J. Biochem. 176,* 103-109
Ricard, J., Soulié, J.-M., Buc, J. & Kellershohn, N. (1988) in *Systems with Learning and Memory Abilities* (Delacour, J. & Levy, J. C. S. eds.), pp. 261-275, Elsevier, North Holland
Schneider, F. W. (1976) *Biopolymers 15,* 1-14
Soulié, J. M., Rivière, M. & Ricard, J. (1988) *Eur. J. Biochem. 176,* 111-117

Chapter 25

Temporal Aspects of the Control of Metabolic Processes

LUIS ACERENZA

A METABOLIC system can be defined as composed of metabolites that are interconverted by enzyme reactions. The change of each metabolite concentration with time (dS_i/dt) depends on the balance between the rates v_j of production and consumption of the metabolite:

$$\frac{dS_i}{dt} = \sum_j n_{ij} v_j, \quad i = 1, \cdots, m \tag{1}$$

in which n_{ij} is the stoicheiometric coefficient of S_i in the reaction j and m is the number of metabolites.

Metabolic control analysis as proposed by Kacser & Burns (1973) and Heinrich & Rapoport (1974) studies the case where the metabolite concentrations are constant in time ($dS_i/dt = 0$ for all i), i.e. the steady state. Most of the contributions to the field, including most of the work described in other chapters of this book, deal with this particular case. Metabolic control analysis of the steady state is principally concerned with the effects of small changes in parameters (concentrations of enzymes, external effectors, etc.) on the steady-state values of the variables (metabolite concentrations and fluxes). In operational terms, this aspect of metabolism may be described by the following basic recipe: "Measure the steady-state value of the variable (reference state). Modify one parameter by a small relative amount. Wait until the system settles to a new steady state. Measure the final steady-state value of the variable." The quantitative description is summarized by the *control coefficient* (Burns *et al.*, 1985):

$$C_p^Y = \frac{\delta Y}{Y} \Big/ \frac{\delta p}{p} \tag{2}$$

where $\delta Y/Y$ is the small relative change in the steady-state value of a particular variable Y induced by the small relative change $\delta p/p$ in the parameter p, when all the other

Luis Acerenza ● Department of Genetics, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK ✥ Permanent address: Departamento de Biofísica y Bioquímica, Facultad de Humanidades y Ciencias, Universidad de la República, Montevideo, Uruguay

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

parameters are maintained at their reference values. In the basic recipe of steady-state control analysis the time is considered implicitly. After the change in a parameter we assume that the system will reach a new steady state. This is an asymptotic state, and therefore we need, in theory, an infinite time to attain it. In practice we wait a "long enough" but finite period of time to reach a good approximation of the new steady-state value. To estimate what is "long enough" one needs to know the order of magnitude of the relaxation time of the variable to the new steady state, but a detailed knowledge of the instantaneous values of the variable during the transition is not required..

The steady-state treatment is a good description for answering some questions, but there are many biological phenomena at different structural levels of organization where the steady-state assumption is not a good approximation. At the molecular level, metabolic systems may exhibit temporal behaviour that ranges from simple monotonic transients to oscillations and deterministic chaos (Higgins, 1967; Higgins *et al.*, 1973; Kohn *et al.*, 1979; Easterby, 1981; Decroly & Goldbeter, 1982; Kohn & Chiang, 1982; Markus *et al.*, 1984; Pachot & Demongeot, 1987; Mizraji *et al.*, 1988; see also Chapter 26 by Markus and Hess in this book). To study the effect of parameters on different variables of a time-dependent metabolic system, control analysis should be extended. We recently developed the basic definitions and relationships to analyse some control features of the instantaneous values of metabolite concentrations of metabolic concentrations and fluxes (Acerenza *et al.*, 1989). Here I shall outline some aspects of this work, and use them to analyse some control properties of time-invariant variables of time-dependent systems.

Returning to eqn. (1), describing the metabolic system, the solutions of this system of differential equations are the values that each S_i can take in time. From these values and the rate equations, the instantaneous fluxes J_j can be calculated. These time courses depend on the values of the parameters. Then, considering a reference time course (generated by a set of reference parameters), we may pose the following question: how are the reference values of a variable modified when one or more parameters are changed at the initial time? In what follows I am interested in one particular change of the parameters. I shall study the case where all the enzyme concentrations are simultaneously altered from their reference values by the same factor α. I shall call this simultaneous change of parameters the *coordinate-control operation*. If E_j are the reference values of the enzyme concentrations, the values after the coordinate-control operation are as follows:

$$E_{j,\alpha} = \alpha E_j \tag{3}$$

I shall use the subscript α to indicate the value of a parameter or variable after the coordinate-control operation. From now on, I assume that the rates v_j are proportional to the corresponding total enzyme concentrations:

$$v_j = E_j f_j \tag{4}$$

where f_j is a function of some metabolite concentrations and parameters, but not of enzyme concentrations and time. Under this hypothesis, if we apply the coordinate-control operation the new rates are given by:

$$v_{j,\alpha} = \alpha v_j \tag{5}$$

and, combining eqns. (1) and (5), the system of differential equations that describes the new situation is as follows:

$$\frac{dS_{i,\alpha}}{d\tau_\alpha} = \sum_j n_{ij} v_j, \quad i = 1, \cdots, m \tag{6}$$

where $\tau_\alpha = \alpha t_\alpha$. As the right-hand sides of eqns. (1) and (6) are identical, the only effect of the coordinate-control operation is to produce a change in the time scale given by

$$\tau = \alpha t \tag{7}$$

Therefore, the solutions of eqns. (1) and (6), $S_i(t)$ and $S_{i,\alpha}(J_\alpha)$ respectively, are identical if τ_α and t are numerically equal. Since $\tau_\alpha = \alpha t_\alpha$ [eqn. (7)], this condition is fulfilled when

$$\tau_\alpha = t/\alpha \tag{8}$$

This means that if for a time t the metabolite concentrations obtained from eqns. (1) have certain values, the solutions of eqns. (6) exhibit the same values at a time t/α:

$$S_{i,\alpha}(t/\alpha) = S_i(t) \tag{9}$$

For the fluxes, we obtain

$$J_{j,\alpha}(t/\alpha) = \alpha J_j(t) \tag{10}$$

When the system approaches a stable steady state, the variables attain approximately constant values in time, and eqns. (9-10) take the following forms:

$$\begin{aligned} S_{i,\alpha}^{ss} &= S_i^{ss} \\ J_{j,\alpha}^{ss} &= \alpha J_j^{ss} \end{aligned} \tag{11}$$

where the superscript ss denotes steady-state values. The results given in eqns. (9-11) are illustrated in Fig. 1 with a simple example. As an immediate consequence of the change in time scale, if one plots the metabolite concentration after the coordinate control operation against time multiplied by α, the resulting curve should coincide with the reference curve. A similar procedure is used as a test for inactivation of an enzyme during assay (Selwyn, 1965; see also Cornish-Bowden, 1979).

In time-dependent systems there are variables which, although having the dimensions of time, have time-invariant values, for example relaxation time, period of oscillation, etc. These time-invariant variables that characterize the time course of the metabolite

$$X_0 \xrightarrow{E_1} S \xrightarrow{E_2} X_2$$

Scheme 1. Model used to obtain the results shown in Fig. 1.

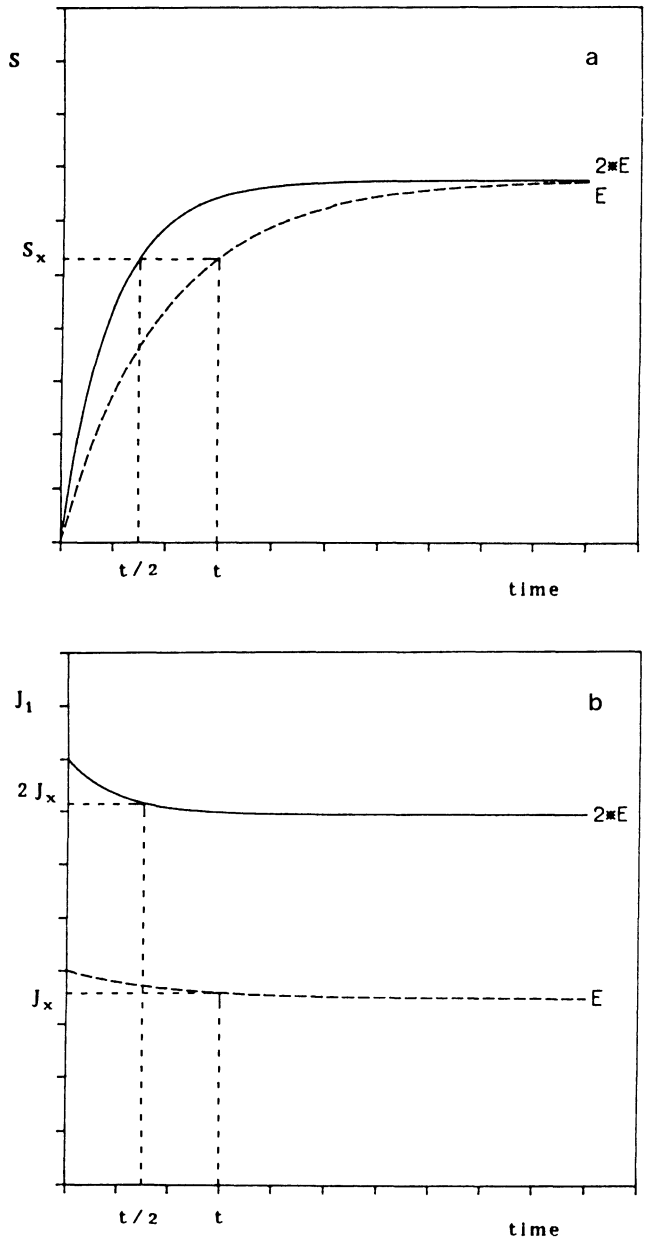
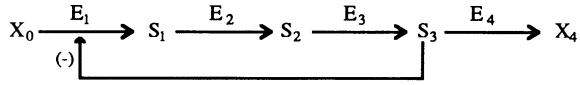


Figure 1. Effect of the coordinate-control operation on the instantaneous values of (*a*) metabolite concentration and (*b*) flux. The model used is shown in Scheme 1, and the rate laws for the two steps are as follows: $v_1 = V_1(X_0 - S/K_1)$ and $v_2 = V_2(S - X_2/K_2)$, with $X_0 = 1$, $X_2 = 0.1$, $K_1 = 1$, $K_2 = 1$. For the reference curve (dashed), $V_1 = 0.2$ and $V_2 = 5$, whereas for the curve after the coordinate-control operation (continuous), $V_1 = 0.4$ and $V_2 = 10$, i.e. in this case $\alpha = 2$. The reference values of the metabolite concentration and flux 1 at time t (arbitrarily chosen) are S_x and J_x respectively. After the coordinate control operation (doubling both V_1 and V_2 at time zero) we obtain the same value of the metabolite concentration, S_x, at time $t/2$ (Fig. 1*a*), but at the same time the flux has twice its reference value (Fig. 1*b*).



Scheme 2. Model used to obtain the results shown in Fig. 2.

concentrations, T, satisfy eqn. (8), i.e.

$$T_\alpha = T/\alpha \tag{12}$$

This is shown for a particular example in Fig. 2, where the variable T is the period of oscillation. Eqn. (12) may be used as the starting point for obtaining the summation relationship for changes in the variable T with the enzyme concentrations E_j. The result is as follows:

$$\sum_j C_{E_j}^T = -1 \tag{13}$$

where

$$C_{E_j}^T = \frac{E_j}{T}\frac{\partial T}{\partial E_j} \tag{14}$$

The general summation relationship given by eqn. (13) has previously been given for particular definitions of transition time (Heinrich & Rapoport, 1975; Torres *et al.*, 1989;

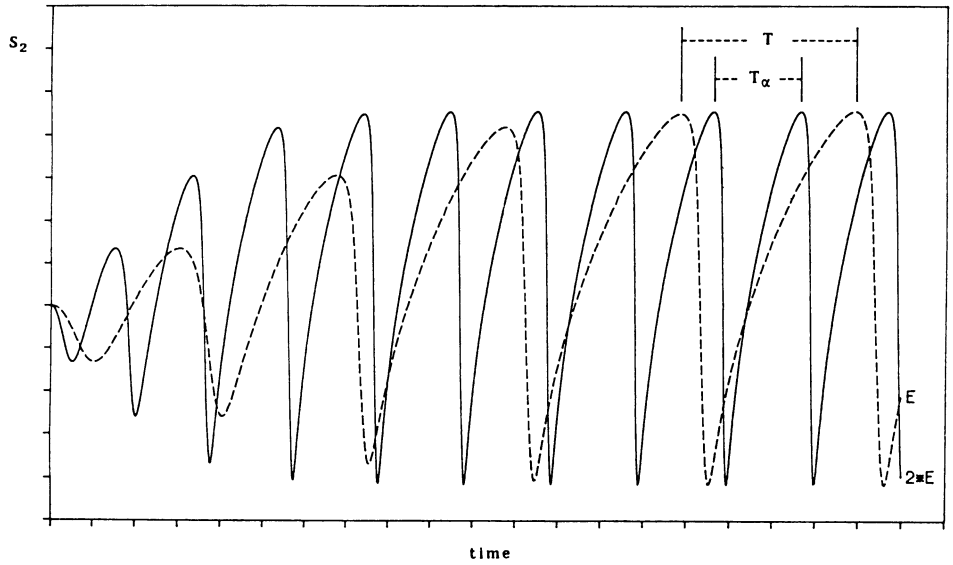


Figure 2. Effect of the coordinate-control operation on the period of oscillation. For the model shown in Scheme 2, the rate law for the first step is $v_1 = V_0X_0/\{X_0 + K_0[1 + L(1 + S_3/K_i)]\}$, and for the other three it is $v_i = VS_i/(K + S_i)$, for i = 1, 2, 3, with parameters X_0 = 5, K_0 = 0.02, K_i = 0.2, L = 10, K = 5. The reference values (dashed curve) of the maximum rates are V_0 = 50 and V = 5. After the coordinate-control operation (continuous line) we use V_0 = 100 and V = 10 (α = 2). One may see from the plots that doubling all the maximum rates causes the period T to decrease to half its reference value, $T_\alpha = T/2$. Numerical simulations were carried out using the program SCAMP (Sauro, 1986).

Meléndez-Hevia *et al.*, 1990). The same relationship applies to the period of oscillation. One quantitative measure for characterizing chaotic behaviour is the Lyapunov exponent λ (see Chapter 26 by Markus and Hess in this book): this quantity has the dimensions of inverse of time, and it may be shown that its reciprocal $1/\lambda$ satisfies eqns. (12-13).

Eqns. (9-13) are some of the consequences of the effect that the coordinate-control operation has on the variables of the metabolic system. In their derivation, some assumptions were made (see Acerenza *et al.*, 1989). One of the most important is that all the rate laws are proportional to the corresponding enzyme concentrations. If the deviations from this assumption are significant (see Chapter 20 by Kacser, Sauro and Acerenza in this book), the equations obtained are not fulfilled. The departures from the expected results may give some information about the control properties of the system. For example if we apply the coordinate-control operation (with a small α) to an oscillatory system (see Chapter 26 by Markus and Hess), and we find that the period does not satisfy eqn. (12), then we may conclude that there are deviations from the assumed hypothesis that contribute significantly to the control of the period. This operation may be applied relatively easily to a biological extract. Therefore, the coordinate-control operation may be used as a simple experimental strategy to gain some insight into the control features of metabolic systems.

Acknowledgements: I wish to thank Dr H. Kacser for continuous encouragement and critical discussion, and the Commission of the European Communities for financial support.

REFERENCES

Acerenza, L., Sauro, H. M. & Kacser, H. (1989) *J. Theor. Biol. 137,* 423-444

Burns, J. A., Cornish-Bowden, A., Groen, A. K., Heinrich, R., Kacser, H., Porteous, J. W., Rapoport, S. M., Rapoport, T. A., Stucki, J. W., Tager, J. M., Wanders, R. J. A. & Westerhoff, H. V. (1985) *Trends Biochem. Sci. 10,* 16

Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics,* pp. 49-51, Butterworths, London and Boston

Decroly, O. & Goldbeter, A. (1982) *Proc. Natl. Acad. Sci. USA 79,* 6917-6921

Easterby, J. S. (1981) *Biochem. J. 199,* 155-161

Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95

Heinrich, R. & Rapoport, T. A. (1975) *BioSystems 7,* 130-136

Higgins, J. (1967) *Ind. Eng. Chem. 59,* 19-62

Higgins, J., Frenkel, R., Hulme, E., Lucas, A. & Rangazas, G. (1973) in *Biological and Biochemical Oscillators* (Chance, B., Pye, E. K., Ghosh, A. M. & Hess, B., eds.), pp. 127-175, Academic Press, New York

Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104

Kohn, M. C., Whitley, L. M. & Garfinkel, D. (1979) *J. Theor. Biol. 76,* 437-452

Kohn, M. C. & Chiang, E. (1982) *J. Theor. Biol. 98,* 109-126

Markus, M., Kuschmitz, D. & Hess, B. (1984) *FEBS Lett. 172,* 235-238

Meléndez-Hevia, E., Torres, N. V., Sicilia, J. & Kacser, H. (1990) *Biochem. J. 265,* 195-202

Mizraji, E., Acerenza, L. & Hernandez, J. (1988) *BioSystems 22,* 11-17

Pachot, P. & Demongeot, J. (1987) in *Contrôle du Métabolisme Cellulaire* (Mazat, J.-P. & Reder, C., eds.), pp. 121-137

Sauro, H. M. (1986) PhD Thesis, Oxford Polytechnic

Selwyn, M. J. (1965) *Biochim. Biophys. Acta 105,* 193-195

Torres, N. V., Souto, R. & Meléndez-Hevia, E. (1989) *Biochem. J. 260,* 763-769

Chapter 26

Control of Metabolic Oscillations: Unpredictability, Critical Slowing Down, Optimal Stability and Hysteresis

MARIO MARKUS and BENNO HESS

CONTROL ANALYSIS can be divided into three main areas. The first deals with systems in the steady state and is discussed in most of the contributions to the present volume. The second deals with time-dependent sensitivity coefficients of the first and second order (Larter *et al.*, 1984; Edelson & Rabitz, 1985), sensitivity densities (Larter *et al.*, 1983, 1984), as well as control and elasticity coefficients (Kohn *et al.*, 1979; see also Chapter 25 by Acerenza in this book). The third is concerned with time-independent coefficients of time-dependent (oscillatory) processes, such as period sensitivities (Larter *et al.*, 1984; Edelson & Rabitz, 1985) or control coefficients, as discussed by Acerenza in Chapter 25 of this book. We shall deal with this third area in the present contribution.

To achieve a theoretical understanding of our experimental observations and the capability of making predictions for experimental falsification or verification, we need to know the number of independent variables that specify the state of the system at any given time (phase space dimension n_p). This dimension n_p is equal to the number of autonomous first order differential equations that describe all observable dynamic processes. It is well known that n_p may be much lower than the total number of variables (in our case chemical concentrations) involved in the system. A classical example was given by the meteorologist Eward Lorenz, who showed that the dynamic properties of the complicated Navier-Stokes equations for fluid flow can in some cases be described by a truncated system with $n_p = 3$ (see, e.g., Schuster, 1984). In the following section we show experimentally that $n_p = 3$ also for glycolytic oscillations. This result makes legitimate a model with three homogeneous first-order differential equations based on detailed kinetic measurements. Below we shall present and compare results obtained from this model and experiments.

Mario Markus and Benno Hess ● Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddamm 201, D–4600 Dortmund 1, Federal Republic of Germany

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

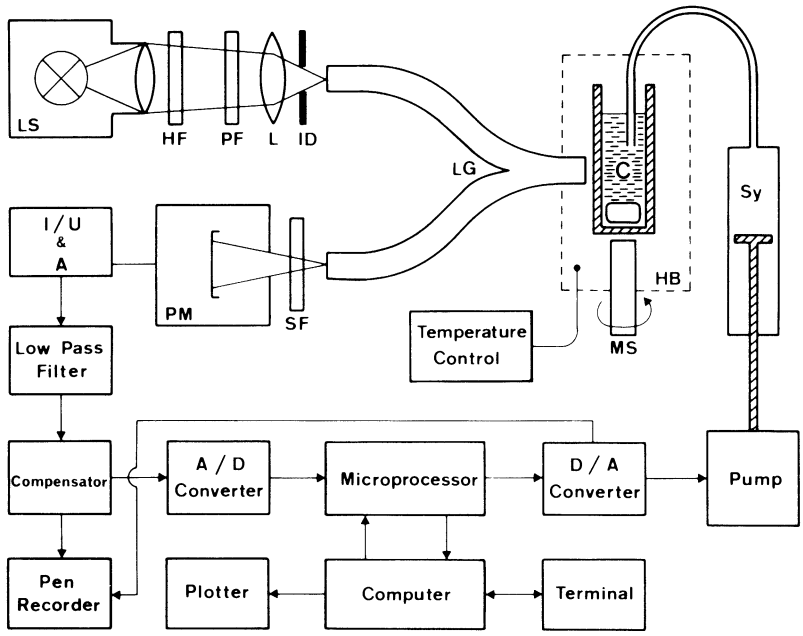


Figure 1. Experimental setup for the measurement of glycolytic oscillations under oscillatory substrate (C: cuvette, HB: heath bath, MS: magnetic stirrer, Sy: syringe, LG: light guides, HF, PF and SF: filters, ID: iris diaphragm, LS:light source, PM: photomultiplier).

How many Independent Variables are there?

For several years measurements of chaotic oscillations have been used as an experimental tool for the determination of the phase space dimension n_p (L'vov *et al.*, 1981; Malraison *et al.*, 1983; Markus & Hess, 1985). The idea behind this is that chaos is an autonomous generator of fluctuations, and that these fluctuations fill up the system phase space as time proceeds. The dimension of this space is n_p and it can be determined by reconstructing trajectories in spaces with increasing dimensions n_r (n_r = 2, 3, 4 ...) from the measured oscillations. Different procedures for the reconstruction of attractors have been proposed (Packard *et al.*, 1980).

The oscillations for the present work were obtained by monitoring the fluorescence F of NADH during a sinusoidal glucose input flux $V_{in} = \bar{V}_{in} + A\sin(\omega_e t)$. The materials and methods are those given by Markus *et al.* (1985*a*). A scheme of the experimental set up is shown in Fig. 1. Fig. 2 shows examples of measured chaotic oscillations. Further experimental results can be found in previous papers (Markus *et al.*, 1984, 1985*ab*).

We reconstructed trajectories with $n_r = 2$ by plotting $F(t)$ against $F(t + \tau)$, where τ is a fixed time delay, and trajectories with $n_r = 3$ by rotating the $F(t)$ - $F(t + \tau)$ plane around the $F(t)$ axis with angular frequency ω_e. Higher-dimensional spaces were constructed using $F(t + 2\tau)$ and $F(t + 3\tau)$ as additional coordinates. Fig. 3 shows an example of a reconstructed attractor with $n_r = 3$.

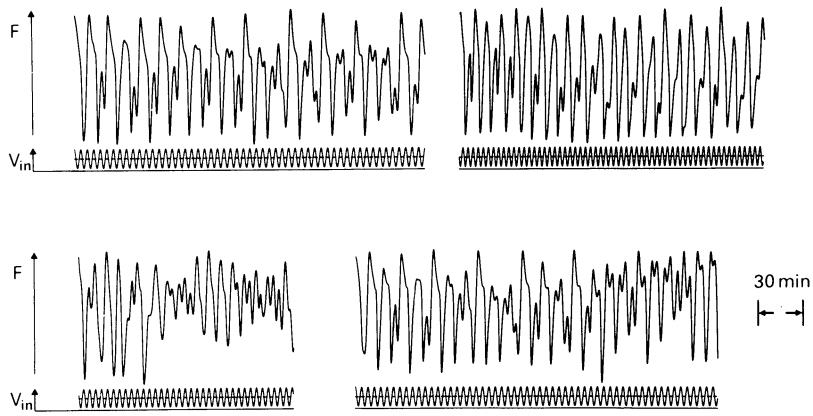


Figure 2. Four measurements of chaotic oscillations (F: NADH fluorescence of the glycolysing yeast extract, V_{in}: glucose input flux).

From the reconstructed trajectories we determined the maximum Lyapunov exponent λ_{max} as a global parameter characterizing the chaotic signal. This quantity is given by

$$\lambda_{max} = \frac{1}{t_{tot}} \sum_{k=1}^{N} \log \frac{L'(t_k)}{L(t_{k-1})} \tag{1}$$

Here t_{tot} is the total observation time, which is divided into N equal intervals. $L(t_{k-1})$ and $L'(t_k)$ are the distances between points of nearby trajectories at times t_{k-1} and t_k (Wolf *et al.*, 1985). λ_{max} quantifies the degree of unpredictability of the chaotic process. In fact,

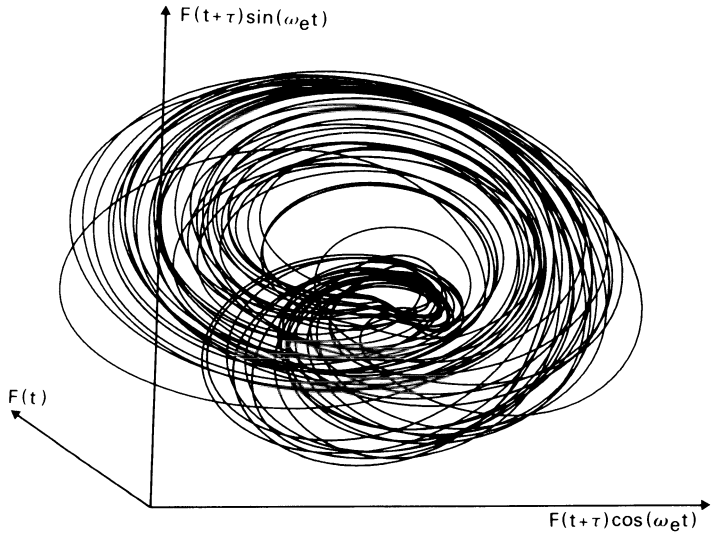


Figure 3. Attractor reconstructed from a measured chaotic oscillatory train.

Table 1. Lyapunov exponents (min^{-1}) for different experiments and phase space dimensions

	n_T: 2	3	4	5
Experiment 1	0.35	0.15	0.14	0.16
Experiment 2	0.28	0.10	0.12	0.12
Experiment 3	0.65	0.39	0.42	0.40
Experiment 4	0.74	0.31	0.30	0.33

any uncertainty in the initial conditions grows in proportion to $\exp(\lambda_{max}t)$ in the time average.

Table 1 gives the value of λ_{max} for four experiments, n_T ranging from 2 to 5. The table reveals that the value of λ_{max} saturates (within numerical errors) for $n_T \geq 3$, indicating that a three-dimensional space is sufficient to embed the trajectories. This result is consistent with a previous work where it was shown that the information dimension determined from measurements also saturates for $n_T \geq 3$ (Markus & Hess, 1985*a*). We have never observed any oscillations that need a higher embedding dimension. In general, the highest embedding dimension is an upper bound for n_p. Thus, in our case, $n_T \geq 3$. On the other hand, chaos can only exist in continuous systems for $n_T \geq 3$. Thus, we easily conclude $n_p = 3$ from our measurements. It should be kept in mind, however, that in other systems the highest embedding dimension (upper bound for n_p) may be higher than 3. In such cases, n_p is obtained using additional diagnostic tools, like the determination of condition-probability-densities by slicing the attractors with hypersheets, as described by Packard *et al.* (1980).

Our experimental result $n_p = 3$ legitimizes the use of a model with three phase variables. The following theoretical results were obtained from such a three-variable model, the parameters of which were derived from detailed kinetic measurements (Markus & Hess, 1984; Hess & Markus, 1985*ab*). In accordance with previous investigations (Markus *et al.*, 1985*b*) we scale the time here by assuming a maximum rate for pyruvate kinase of 1.4 mM/min.

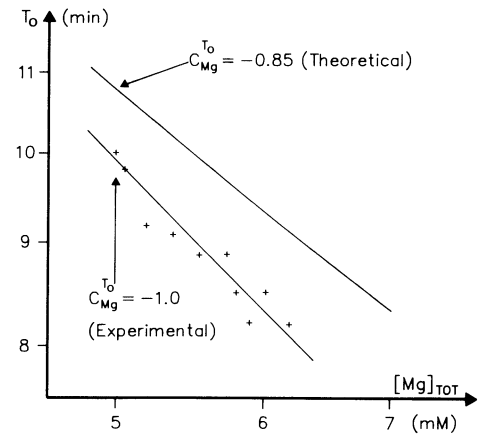


Figure 4. Period T_0 of autonomous glycolytic oscillations versus total magnesium concentration.

Experimental versus Theoretical Results

Control of Autonomous Oscillations. The autonomous oscillation period, i.e. the period at constant glucose input flux, was determined theoretically and experimentally as a function of total magnesium concentration. The results are shown in Fig. 4. In the experiments, $[Mg]_{tot}$ was varied by adding magnesium to the reaction mixture. For drawing the crosses in Fig. 4, we assumed that $[Mg]_{tot}$ = 5 mM in the yeast extract without magnesium addition. The plot is double-logarithmic allowing the straighforward determination of the control coefficient :

$$C_{Mg}^{T_0} = \frac{\partial \ln T_0}{\partial \ln [Mg]_{tot}} \qquad (2)$$

where we have used the nomenclature of Burns *et al.* (1985).

The experimental value $C_{Mg}^{T_0}$ = -1 unveils the control of magnesium, which had been neglected in previous models (e.g. by Boiteux *et al.*, 1975). The model considered here includes magnesium by taking into account its effect on the sink enzyme pyruvate kinase in accordance with kinetic measurements (Markus *et al.*, 1980; Boiteux *et al.*, 1983).

Entrainment, Quasiperiodicity and Unpredictability. Measurements of entrainment of glycolysis by a periodic input flux were first reported by Boiteux *et al.* (1975) who observed multiplication of the input period by factors 1, 2 and 3. Later on, also multiplication of the input period by factors 4, 5, 7 and 9 as well as quasiperiodicity (frequency mixing) and chaos (unpredictability) were found (Markus *et al.*, 1984, 1985*ab;* Hess & Markus, 1987).

Fig. 5*a* summarizes all our experiments results obtained upon variation of the dimensionless quantities $A/\bar{V}_{in}$ and ω_e/ω_o, where $\omega_o = 2\pi/T_o$ is the frequency of the autonomous oscillations. Each cross on the figure corresponds to one measured oscillatory train. For comparison, Fig. 5*b* shows the results obtained from our model (Markus & Hess, 1984). In Figs. 5*a* and 5*b*, the numbers indicate the periodic-multiplication-factor. Chaos is reached by three classical routes : (*a*) Period-doubling cascades (the so-called Feigenbaum route) starting from a period-multiplication-factor 2, e.g. at constant $A/\bar{V}_{in}$ and increasing ω_e/ω_o (arrow pointing to the right on fig. 5*b*); (*b*) intermittency (the so-called Manneville-Pomeau route) starting from a period-multiplication factor of 3, e.g. at constant $A/\bar{V}_{in}$ and decreasing ω_e/ω_o (arrow pointing to the left on Fig. 5*b*); and (*c*) breakdown of a torus (the so-called Ruelle-Takens route) starting from quasiperiodicity, e.g. at constant ω_e/ω_o and increasing $A/\bar{V}_{in}$ (arrow pointing upwards on Fig. 5*b*).

Critical Slowing Down and Optimal Stability. We showed above how the degree of unpredictability of a chaotic process can be quantified by the maximum Lyapunov exponent λ_{max}. For a periodic process, λ_{max} can be determined after a slight perturbation of the trajectory. One then obtains negative values of λ_{max}, indicating that small perturbations decay proportionally to $\exp(\lambda_{max}t)$. For simulated periodic oscillations, λ_{max} can readily be calculated by linear perturbation analysis (see Hess & Markus, 1984); results are shown in

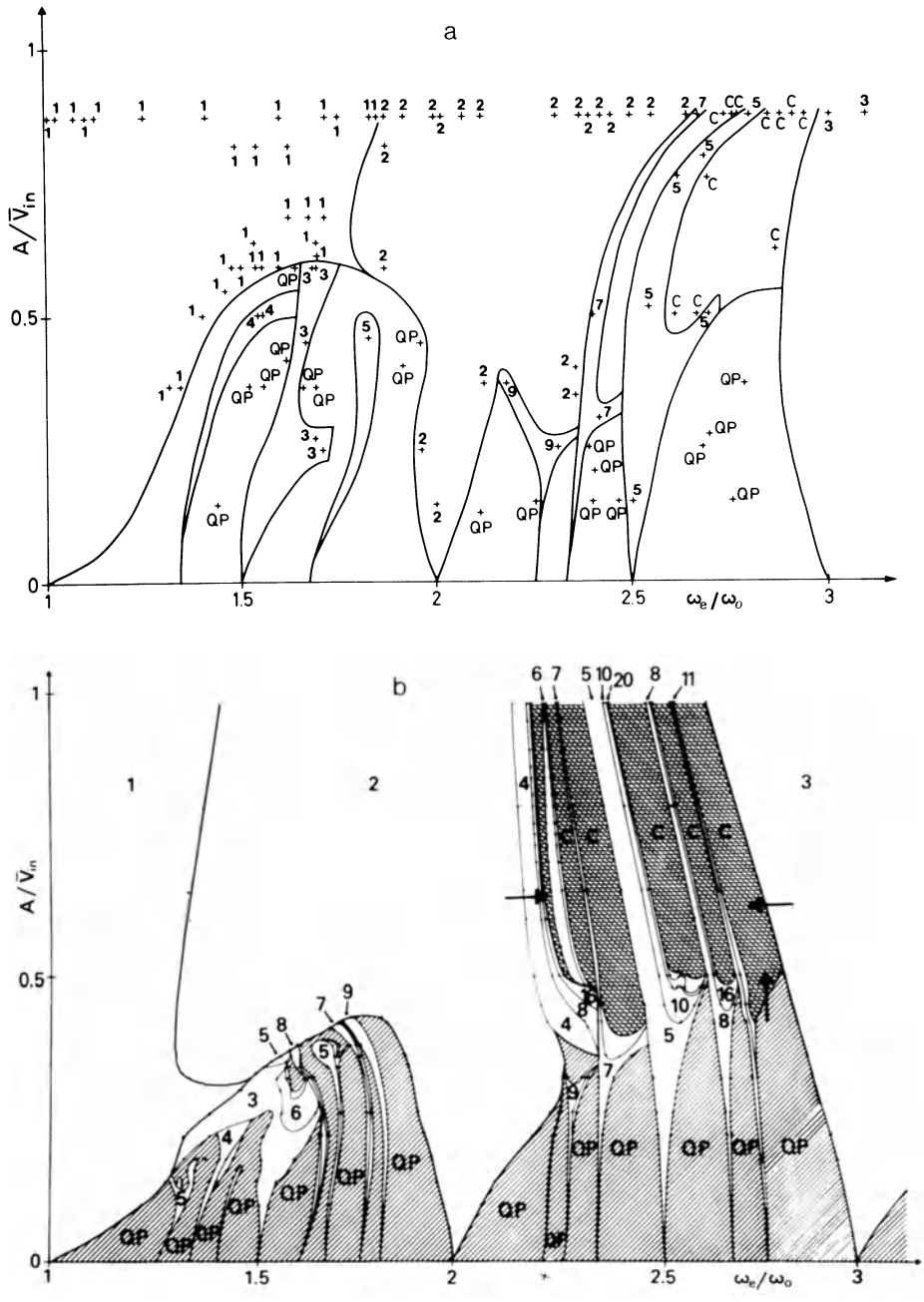


Figure 5. Oscillatory response of glycolysis under periodic glucose input flux. *A*: amplitude of the input flux V_{in} = mean input flux; ω_e: frequency of the input flux; ω_o: frequency at constant input flux; QP: quasi-periodicity; C: chaos. The numbers indicate the factor by which the input period is multiplied to yield the response period. (*a*) Experimental responses: each cross corresponds to one measured oscillatory train. (*b*) Theoretical responses (the three arrows at the upper right side indicate three different routes to chaos).

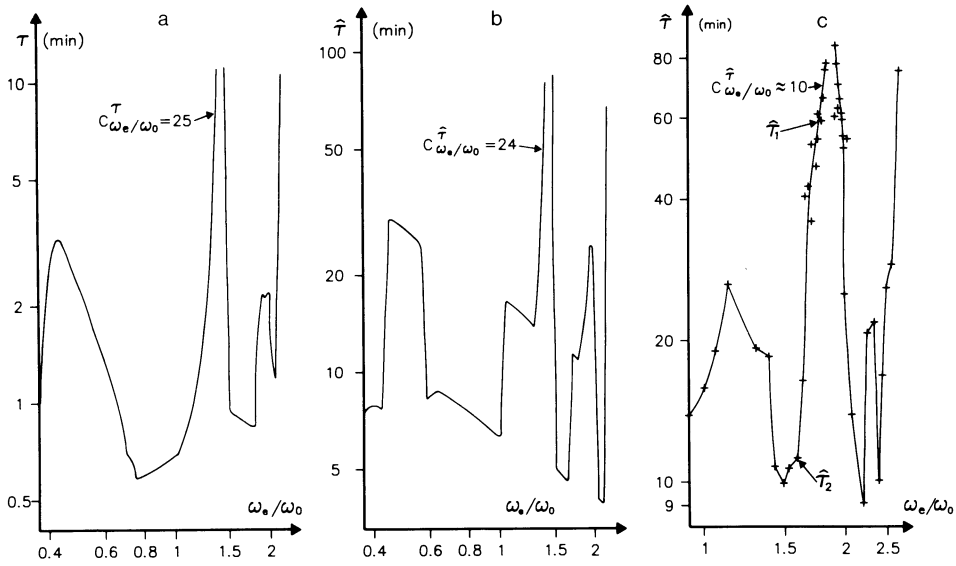


Figure 6. (*a*) Theoretical time of relaxation τ to glycolytic oscillations after small, exponentially decaying perturbations. (*b*) Theoretical times of relaxation τ to glycolytic oscillations after changing from constant to oscillatory glucose input flux. (*c*) Experimental values of $\hat{\tau}$ ($\hat{\tau}_1$ and $\hat{\tau}_2$ correspond to the experiments shown in Fig. 7)

Fig. 6*a*, where we have plotted the relaxation time $\tau = \lambda_{max}^{-1}$ versus ω_e/ω_0 at constant $A/\overline{V}_{in} = 1$. The figure shows singularities ($\tau \rightarrow \infty$) which correspond to the so-called "critical slowing down" (Heinrichs & Schneider, 1981) and occur at bifurcation points. Here, critical slowing down occurs at the transitions of period-multiplication factors from 1 to 2, and from 2 to 4. In the vicinity of critical slowing down, the control coefficients

$$C^{\tau}_{\omega_e/\omega_0} = \frac{\partial \ln \tau}{\partial \ln(\omega_e/\omega_0)} \tag{3}$$

attain arbitrarily high absolute values. For example, $C^{\tau}_{\omega_e/\omega_0} = 25$ at the point indicated by an arrow in Fig. 6*a* (Note that the plots in Fig. 6 are double logarithmic, so that the control coefficients can be readily obtained by the slopes of the curves). Besides singularities, Fig. 6*a* shows minima of τ. At these minima, relaxation of the system after a perturbation is extremely fast, and the relaxation time is relatively insensitive to a change in the external condition ω_e/ω_0 (optimal stability).

In contrast to model systems, it is extremely difficult to obtain τ experimentally in oscillating glycolysis, since a decay — small enough to be described by linear perturbation analysis, and thus comparable to the theoretical results shown in Fig. 6*a* — is easily overshadowed by noise or systematic drifts. Therefore, we introduce a modified relaxation time $\hat{\tau}$ for large (in general non-exponentially decaying) perturbations.

We define $\hat{\tau}$ as the length of the time interval $[t_a, t_e]$, where $V_{in} = \overline{V}_{in} = \text{const.}$ (i.e.

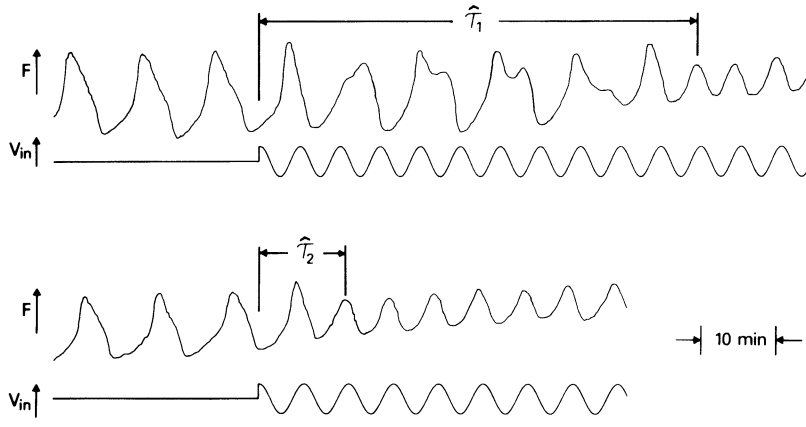


Figure 7. Long (upper, $\hat{\tau}_1$) and short (lower, $\hat{\tau}_2$) times of relaxation to entrained oscillations.

$A = 0$) for $t < t_a$, F has minimum at $t = t_a$, $V_{in} = \overline{V}_{in} + A\cos[\omega_e(t - t_a)]$ for $t \geq t_a$, and t_e is the time at which F reaches its first maximum such that the period deviates less than 10% from its final value. In other words, $\hat{\tau}$ indicates how fast the system recovers from an infinitely long perturbation given by $V_{in} = \overline{V}_{in} = \text{const}$.

In contrast to τ, the modified relaxation time $\hat{\tau}$ is easily accessible experimentally, as illustrated in Fig. 7. This figure shows a long and a short modified relaxation time ($\hat{\tau}_1$ and $\hat{\tau}_2$). $\hat{\tau}_1$ occurs near critical slowing down, whereas $\hat{\tau}_2$ occurs near optimal stability. In both cases, the system relaxes towards entrainment by the fundamental frequency (period-multiplication factor of 1).

Theoretically, $\hat{\tau}$ was determined from our model (Markus & Hess, 1984) by considering that the phosphoenolpyruvate concentration displays a dynamic behaviour analogous to that of [NAD], which is indicated by the measured $F_{max} - F$ because of the conservation of [NADH] + [NAD$^+$] (Markus *et al.*, 1985*a*). We thus determined $\hat{\tau}$ theoretically from the time evolution of the phosphoenolpyruvate concentration applying the rule stated above for $F(t)$, except that we considered minima and maxima of the phosphoenolpyruvate concentration instead of maxima and minima respectively of F. Fig. 6*b* shows the theoretically calculated $\hat{\tau}$ versus ω_e/ω_o for the same conditions as Fig. 6*a*. Comparison of Figs. 6*a* and 6*b* shows that $\hat{\tau}$ displays (in a somewhat distorted fashion) the main properties of τ. The experimentally determined $\hat{\tau}$ are shown in Fig. 6*c*. This figure shows both optimal stability and critical slowing down and is comparable to the theoretical predictions of Fig. 6*b*.

Non-uniqueness of Control: Hysteresis. Metabolic control may depend on the previous history of a system (hysteresis). For oscillating systems, this phenomenon has been demonstrated theoretically with different models (Decroly & Goldbeter, 1982; Markus & Hess, 1984). Hysteresis implies the existence of two or more dynamic modes for the same set of external conditions. The existence of two, three and four modes is called birhythmicity, trirhythmicity and tetrarhythmicity respectively.

Experimentally, we found birhythmicity in glycolytic oscillations at constant glucose

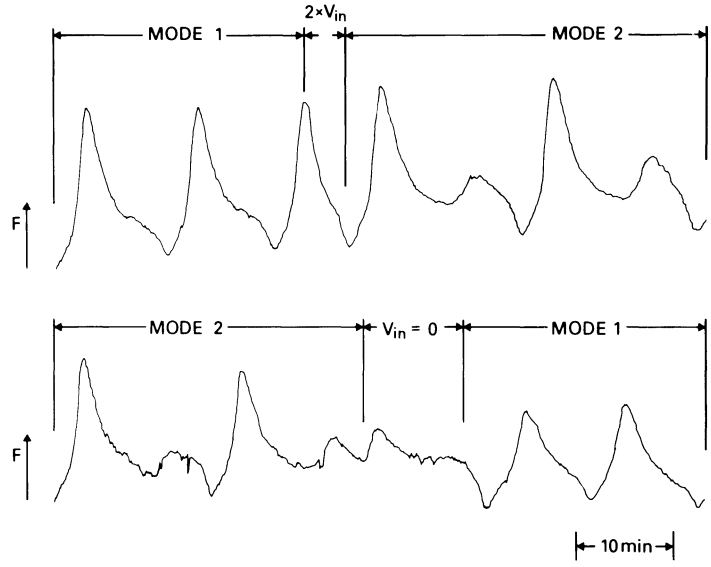


Figure 8. Switching from mode 1 to mode 2 by a transitory doubling of the glucose input flux V_{in} (upper), and switching from mode 2 to mode 1 by a transitory shut-off of the input flux (lower). The conditions for mode 1 and mode 2 in the upper and in the lower curves are identical (birhythmicity). Input flux for both modes: V_{in} =0.22 mM/min = const.

input flux ($A = 0$), as shown in Fig. 8. In this figure, mode 1 (the commonly observed mode, which is characterized by equally large maxima of F) and mode 2 (a mode that had been reported by Hess, 1966, and is characterized by alternating small and large maxima of F) exist for the same external conditions. Mode 1 is obtained here by increasing V_{in} from 0 to 0.22 mM/min, while mode 2 is obtained by reaching V_{in} = 0.22 mM/min from larger values. Switching between coexisting modes has been described theoretically (Markus & Hess, 1984). In the present experimental work, the coexisting modes could be switched into one another as shown in Fig. 8. In the upper part of this figure, mode 1 is switched into mode 2 by doubling V_{in} temporarily. In the lower part of Fig. 8, mode 2 is switched into mode 1 by temporarily suppressing the glucose input. Concerning the length of observation times, we found here that mode 1 is as long lived as the oscillatory extract, but mode 2 never remains longer than 12 oscillatory maxima, ending with an abrupt transition into mode 1. In view of such an abrupt transition, we assume that the basin of attraction of mode 2 in phase space is extremely small, so that fluctuations or a small systematic drift drive the system from the attractor of mode 2 into the basin of mode 1, where it rapidly relaxes to the corresponding attractor (see Markus & Hess, 1985*b*, 1986).

Conclusions

We have shown that the interplay between experiments and quantitative modelling can lead to considerable insight in the control of dynamic metabolic processes.

There has been much discussion about whether chaotic biochemical oscillations are physiological, pathological or just an exotic *in vitro* curiosity. Here, we do not engage in this discussion, but deal with chaos as a valuable tool for the determination of the number n_p of independent variables that describe the system. A quantitative model with this n_p is in excellent agreement with experimental results, in particular, we measured period-control by magnesium, as well as entrainment, quasi-periodicity, chaos, critical slowing down and optimal stability under conditions close to those predicted by the model.

In the present work, hysteresis was found experimentally for constant input flux. However, our model predicts this phenomenon for oscillatory input. The model of Decroly & Goldbeter (1982) predicts hysteresis at constant input, but requires two positive enzymic feedback loops, and it is questionable if such an assumption is applicable to glycolysis. This point is open to further research.

It is interesting that hysteresis has also been found for a single enzyme which exists under different conformations that slowly isomerize during turnover (see Chapter 24 by Ricard *et al.* in this book). Such an enzyme behaves as a biosensor that is able to detect both a concentration and the direction of concentration change. Functionally, this phenomenon is comparable to the behaviour of glycolysis as described here, and it may be of general biological significance (Hess & Markus, 1985*ab;* Ricard & Buc, 1988). It should be kept in mind that sensing of change through glycolysis is possible in spite of our observation that one of the coexisting modes breaks down after a number of oscillations.

Acknowledgements: We thank Jörg Schütze for assistance in the laboratory. This work was supported by the Stiftung Volkswagenwerk.

REFERENCES

Acerenza, L., Sauro, H. M. & Kacser, H. (1989) *J. Theor. Biol.*, in press

Boiteux, A., Goldbeter, A. & Hess, B. (1975) *Proc. Natl. Acad. Sci. USA 72,* 3829-3833

Boiteux, A., Markus, M., Plesser, Th. Hess, B. & Malcovati, M. (1983) *Biochem. J. 211,* 631-640

Burns, J. A., Cornish-Bowden, A., Groen, A.K., Heinrich, R., Kacser, H., Porteus, J. W., Rapoport, S. M., Rapoport, T. A., Stucki, J. W., Tager, J. M., Wanders, R. J. A. & Westerhoff, H. V. (1985) *Trends Biochem. Sci. 10,* 16

Decroly, O. & Goldbeter, A. (1982) *Proc. Natl. Acad. Sci. USA 79,* 6917-6921

Edelson, D. & Rabitz, H. (1985) in *Oscillations and Travelling Waves in Chemical Systems* (Field, R. J. & Burger, M., eds.), pp. 193-222, J. Wiley, New York

Heinrichs, M. & Schneider, F. W. (1981) *J. Chem. Phys. 85,* 2112-2116

Hess, B. (1966) *Studia Biophysica s1*, s41-s63

Hess, B. & Markus, M. (1984) in *Synergetics — From Microscopic to Macroscopic Order* (Frehland, E., ed.), pp. 6-16, Springer-Verlag, Berlin

Hess, B. & Markus, M. (1985*a*) in *Temporal Order* (Rensing, L. & Jaeger, N.I., eds.), pp. 179-190, Springer-Verlag, Berlin

Hess, B. & Markus, M. (1985*b*) *Ber. Bunsenges. Phys. Chem. 89*, 642-651

Hess, B. & Markus, M. (1987) *Trends Biochem. Sci. 12,* 45-48

Kohn, M.C., Whitley, L.M. & Garfinkel, D. (1979) *J. Theor. Biol. 76,* 437-452

Larter, R., Rabitz, H. & Kobayashi, M. (1983) *J. Chem. Phys. 79,* 692-707

Larter, R., Rabitz, H. & Kramer, M. (1984) *J. Chem. Phys. 80,* 4120-4128

L'vov, V.S., Predtechensky, A.A. & Chernikh, A.I. (1981) *Soviet Physics JETP 53,* 562-581
Malraison, G., Atten, P., Bergé, P. & Dubois, M. (1983) *J. Phys. Lett. 44,* 897-902
Markus, M., Plesser, Th. Boiteux, A., Hess, B. & Malcovati, M. (1980) *Biochem. J. 189,* 421-433
Markus, M. & Hess, B. (1984) *Proc. Natl. Acad. Sci. USA 81,* 4394-4398
Markus, M., Kuschmitz, D. & Hess, B. (1984) *FEBS Lett. 172,* 235-238
Markus, M. & Hess, B. (1985*a*) in *Temporal Order* (Rensing, L. & Jaeger, N.I., eds.), pp. 191-193, Springer-Verlag, Berlin
Markus, M. & Hess, B. (1985*b*) *Arch. Biol. Med. Exp. 18,* 261-271
Markus, M., Müller, S. C. & Hess, B. (1985*a*) *Ber. Bunsenges. Phys. Chem. 89,* 651-654
Markus, M., Kuschmitz, D. & Hess, B. (1985*b*) *Biophys. Chem. 22,* 95-105
Markus, M. & Hess, B. (1986) in *Dynamics of Biochemical Systems* (Damjanovich, S., Keleti, T. & Trón, L., eds.), pp. 11-25, Akadémiai Kiadó, Budapest
Packard, N. H., Crutchfield, J. P., Farmer, J. D. & Shaw, R. S. (1980) *Phys. Rev. Lettt. 45,* 712-716
Ricard, J. & Buc, J. (1988) *Eur. J. Biochem. 176,* 103-109
Schuster, H. G. (1984) *Deterministic Chaos,* Physik-Verlag, Weinheim
Wolf, A., Swift, J. B., Swinney, H. L. & Vastano, J. A. (1985) *Physica 16D,* 285-317

Part VI

EXPERIMENTAL APPLICATIONS

Chapter 27

A New Method for Estimating Enzyme Activity and Control Coefficients *in vivo*

BARBARA E. WRIGHT and KATHY R. ALBE

AN UNDERSTANDING of metabolism *in vivo* must, of course, be based upon a systems analysis that includes an examination of the role and relationships of all the variables involved. These include the concentrations of substrates, products, and effectors as well as enzyme kinetic mechanisms, constants and enzyme activity. Current research in areas such as biochemical differentiation, aging, biotechnology, etc., is focussed primarily on the regulation of metabolism by enzyme activity. In fact, metabolites are generally *more* important than enzymes in controlling reaction rates. As we shall be discussing the citric acid cycle it is appropriate to quote Krebs (1957): "The average half-life of the acids in the tricarboxylic acid cycle ... is a few seconds. The amounts of enzyme in the tissue are sufficient to deal with the intermediates as soon as they arise: in other words, the amount of available substrate is the factor limiting the rate at which the intermediary step proceeds." And yet, the recent literature on metabolic regulation *in vivo* describes the role of metabolites by such passive and mysterious words as "elasticity," while enzymes are given credit as the force behind "control coefficients." Reactions are often referred to by the name of the enzyme catalyst, and the rate of a reaction *in vivo* is often equated with the activity of the enzyme. Some of this prejudice stems from *in vitro* studies, in which enzymes must be diluted to the point at which they limit the rate of the reaction being measured, while substrates must be present at levels greatly exceeding their *in vivo* concentration.

We find some of the definitions currently in use confusing. With all due respect to the other contributors to this book, we are going to be simple-minded and even-handed in dealing with enzymes and metabolites. We shall first name the stimulus and then the response; that is, we shall speak of enzyme on flux coefficients, enzyme on metabolite coefficients, and metabolite on flux coefficients.

A theory must, of course, originate with experimental observations. It is an extrapolation — a generalization or prediction about the probable behaviour of a system based upon the

Barbara E. Wright and Kathy R. Albe ● Division of Biological Sciences, University of Montana, Missoula, Montana 59812, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

previous behaviour of a similar system. Subsequent experiments may falsify the hypothesis or strengthen it, by demonstrating its predictive value. As we understand it, two generalizations have been proposed as applicable to all metabolic systems *in vivo*, under steady state conditions, regardless of their complexity. First, the sum of the coefficients of all enzymes on flux through the system is one; and second, the sum of the coefficients of all enzymes on each metabolite is zero (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). These "summation properties" are based upon the theoretical analysis of relatively simple systems. They have rarely been tested in complex metabolic systems *in vivo* under steady state conditions. In fact, a major problem lies in the difficulty of testing these theories. There are very few specific, non-invasive techniques with which to probe metabolism *in vivo*. Realistic dynamic models may come very close to being valid test systems. Our analysis of the citric acid cycle and carbohydrate metabolism in *Dictyostelium* indicates that, in our highly data-based model systems, the so called "summation properties" do not hold. We have found that the sum of the coefficients of all enzymes on flux can vary from 0.2 to 2.0, and that these coefficients are a function of the ratio of enzyme activity to flux through the system, and the range of enzyme activity values chosen with which to calculate these coefficients.

In *Dictyostelium,* endogenous protein is used as an energy source. The amino acid composition of the protein being used does not change, suggesting that all protein species, including most enzymes, are degraded (Wright & Butler, 1987). In fact, about half the enzymes studied in this system are subject to rapid proteolytic inactivation in extracts, in the absence of stabilizing substrates or protease inhibitors (Wright & Dahlberg, 1968). We know a lot about flux through and into the citric acid cycle (Kelly *et al.*, 1979*ab*). Knowing the rate of protein degradation and the pathways by which specific amino acids are converted to specific cycle intermediates, we can calculate flux into the cycle at specific points. From O_2 consumption, we can calculate flux through the cycle. To confirm these and other rate determinations, we have constructed steady state models, based on specific radioactivity data; these models also provide information on intra- and extra-mitochondrial metabolic compartments. With a specific radioactivity curve-matching program, we modelled data obtained under steady state conditions from exposing cells to tracer levels of radioactive amino acids and determining the specific radioactivity of citric acid cycle intermediates with time (Kelly *et al.*, 1979*ab*). The configuration, fluxes, and metabolite concentrations in this model were used in the construction of a more complex steady state model which includes enzyme mechanisms and constants. The program used for constructing this model is called METASIM (Wright & Kelly, 1981). We have excluded the extra-mitochondrial pools in this model, which is shown in Fig. 1. All of the enzymes catalysing the reactions which are encircled have been purified from *Dictyostelium* and characterized. Flux through the cycle is between 2 and 3 μmol min^{-1} ml^{-1} mitochondrial volume. As flux through this system is cyclic, choosing the most representative reaction is somewhat arbitrary. We chose reactions 13 and 3 for a number of our tables.

The *Dictyostelium* system is unusual in that endogenous protein is used as the energy source. In a system using only amino acids as the source of cycle intermediates, more amino

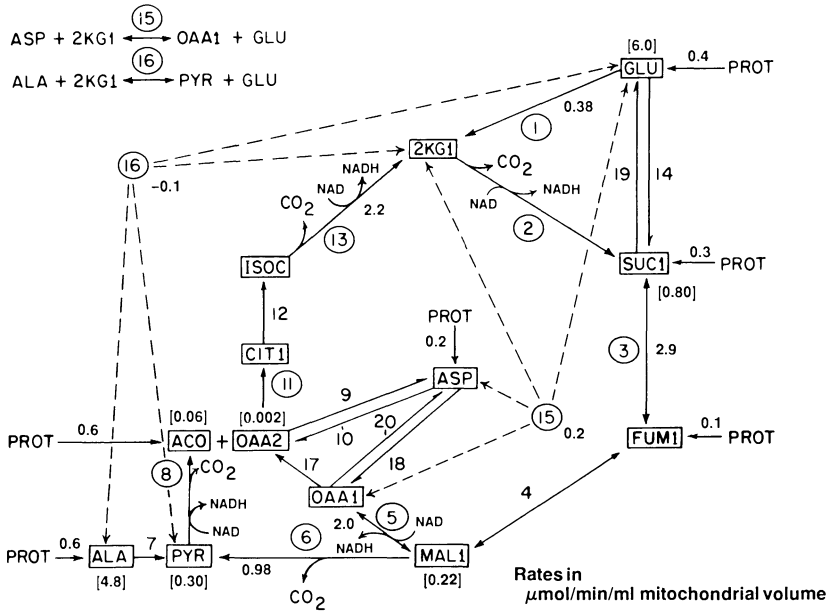


Figure 1. A steady state model of the citric acid cycle in *Dictyostelium*

acids are converted to four- and five-carbon intermediates than to the two-carbon units of acetyl-CoA. For the citrate synthase reaction to operate and maintain steady state levels of cycle intermediates in this system, the flux of acetyl-CoA into the cycle must equal that of oxaloacetate. Therefore, a pathway is required which converts "excess" four- or five-carbon intermediates to acetyl-CoA. This requirement is met by malic enzyme (reaction 6), in which malate is decarboxylated to yield pyruvate which is in turn decarboxylated to form acetyl-CoA via the pyruvate dehydrogenase complex. This pathway has been shown to occur in *Dictyostelium,* and both of these enzymes have been purified and kinetically characterized (Kelleher *et al.*, 1979; Butler *et al.*, 1985). Malic enzyme is allosteric, being positively affected by aspartate, glutamate, succinate and fumarate. Note the differences in metabolite concentrations in this system: acetyl CoA is 0.06 mM, and oxaloacetate is 0.0025 mM. The competition for malate between oxaloacetate and acetyl CoA synthesis is critical in the balance between these two substrates of citrate synthesis. Of all the cycle intermediates, succinate is present at the highest concentration: 0.8 mM. The amino acids included in our analysis are present at levels at least an order of magnitude higher than that of most of the cycle intermediates. The role of the transaminases, catalysing reactions 15 and 16, is complex, as indicated by the dashed lines.

The METASIM program contains 25 different enzyme mechanisms. Input for the model in Fig. 1 includes flux, metabolite concentrations, appropriate enzyme mechanisms and constants for each reaction. For example, the kinetic expression for the rate v of the reaction catalysed by isocitrate dehydrogenase is that for dead-end competitive inhibition:

$$v = \frac{V_{max}[\text{isocitrate}][NAD^+]}{K_{NAD^+}[\text{isocitrate}] + K_{\text{isocitrate}}\left(1 + {}^{[NADH]}/_{K_{NADH}}\right)[NAD^+] + [\text{isocitrate}][NAD^+]}$$

The V_{max} for this enzyme in crude extracts was determined and found to be 5.0 mM/min, based on mitochondrial volume. Using this value in the equation, together with the kinetic constants and known concentrations of isocitrate, NAD^+ and NADH, the rate is calculated to be 0.04 mM/min. However, the rate determined *in vivo* is 2.2 mM/min. Such discrepancies have been found for most of the enzymes which have been purified and characterized in the citric acid cycle and in carbohydrate metabolism. In Table 1, the rate calculated using V_{max} is compared to the rate determined *in vivo* for 23 reactions in the citric acid cycle and carbohydrate models. In general, rates based on V_{max} exceed rates determined directly *in vivo*, that is, more enzyme activity is assayed *in vitro* than is active *in vivo*. The two striking exceptions are 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase, both of which are membrane-bound enzyme complexes, which might be expected to have low recoveries and to fall apart in the preparation of cell extracts. There are a number of possible explanations for observing excess enzyme *in vitro*. Protein is used as an energy source in this system and can be used under nutritional stress in many systems, including our own.

Table 1. A comparison of reaction rates determined *in vivo* with rates calculated from V_{max} values measured with the isolated enzymes

Enzyme	Rate *in vivo*	Calculated rate	Ratio
Isocitrate dehydrogenase	2.20	0.04	0.02
Glutamate dehydrogenase	0.38	0.98	2.6
2-Oxoglutarate dehydrogenase	2.49	0.0014	0.00056
Malic enzyme	1.00	1.71	1.8
Succinate dehydrogenase	2.88	187.8	65.2
Malate dehydrogenase	2.01	12.4	6.2
Citrate synthase	2.20	145.7	66.2
Pyruvate dehydrogenase	1.64	0.0074	0.0045
Aspartate transaminase	0.18	0.48	2.7
Alanine transaminase	-0.10	-0.16	1.6
Glycogen phosphorylase	-0.20	-0.16	0.8
Glucokinase	0.01	0.08	8.0
UDPglucose pyrophosphorylase	0.22	0.37	1.7
Glycogen synthase	0.13	0.83	6.4
Trehalose 6-phosphate synthase	0.009	0.01	1.1
Glucose 6-phosphate dehydrogenase	0.001	1.0	1000.0
6-Phosphogluconate dehydrogenase	0.0009	0.009	10.0
Phosphoglucose isomerase	0.03	6.2	206.0
Uridine phosphorylase	0.01	0.35	35.0
5'-AMP nucleotidase	0.02	2.4	120.0
Glucose 6-phosphatase	0.01	0.006	6.0
Phosphofructokinase	0.01	0.53	53.0
Cell wall glycogen synthase	0.02	0.006	0.3

Table 2. Effects of enzyme activities from 0.5 to 2 times their model values on the fluxes through the reactions they catalyse

Enzyme	Flux control coefficient	V_{vivo} (mM/min)	V_{vivo}/flux
Glutamate dehydrogenase	0.99	2.5	6.5
Aspartate transaminase	0.87	7.8	43.1
Succinate dehydrogenase	0.69	3.3	1.1
Alanine transaminase	0.66	5.2	52.0
Malic enzyme	0.58	2.8	28.0
Malate dehydrogenase	0.18	97.0	48.2
Citrate synthase	0.14	9.1	4.1
2-Oxoglutarate dehydrogenase	0.07	7800.0	3153.0
Fumarase	0	26.0	9.3
Pyruvate dehydrogenase	0	275.0	168.0
Isocitrate dehydrogenase	0	298.0	135.0

Due to the general vulnerability of most proteins to proteolytic attack, excessive enzyme protein concentrations may be essential in order to ensure adequate catalytic activity in times of stress. Another explanation may be that a large fraction of enzyme activity assayed *in vitro* in dilute solution is actually inactive *in vivo*, or unavailable to substrate, due to enzyme-bound product or inhibitor. For example, in muscle, a substantial part of glyceraldehyde-3-phosphate dehydrogenase exists bound to 3-phosphoglycerate (Bloch *et al.*, 1971). Enzymes have also been found to serve structural roles (Wistow *et al.*, 1987).

The observation that more enzyme activity is assayed *in vitro* than is active *in vivo* could also reflect some kind of "channelling." For example, enzymes may exist in high local concentrations *in vivo*, serving as a "buffer" to trap and maximally utilize limiting substrates. If so, all active sites may not be occupied by substrate. However, in dilute cell extracts and in the presence of saturating substrate levels, all active sites would be assayed and V_{max} would exceed enzyme activity *in vivo*. In any event, regardless of the underlying reasons, it would seem unwise to make the leap of faith that *in vitro* enzyme activities are applicable to metabolism *in vivo*. Our observations cast serious doubt upon this assumption.

In view of the fact that V_{max} values do not accurately reflect enzyme activity in vivo, we calculate this activity as the only unknown in each enzyme kinetic expression. For example, V_{max} was calculated for isocitrate dehydrogenase, knowing the reaction rate, kinetic constants and metabolite concentrations. This value, here[1] called V_{vivo}, was 298 mM/min. The term V_{vivo} is used to distinguish this calculated value from the experimentally measured value, V_{max}. These V_{vivo} values are used to determine enzyme on flux and enzyme on metabolite coefficients. The coefficient is the slope obtained by plotting changes in the logarithm of the rate or metabolite concentration against changes in the logarithm of the enzyme activity or metabolite concentration. An example is given in Table 2, in which coefficients are given for the effect of each enzyme on the rate of the reaction it catalyses.

[1]The abbreviated symbol V_v is used in the figures and was used in previous publications.

Table 3. Effect of V_{vivo}/flux on flux control coefficients

Enzyme	V_{vivo} (mM/min)	[Substrate] (mM)	Flux (mM/min)	V_{vivo}/flux	Flux control coefficient
Fumarase	26.0	0.04	2.8	9.3	0
	2.8	0.7	2.3	1.2	0.55
Succinate dehydrogenase	3.28	0.8	2.88	1.1	0.69
	16.4	0.04	3.89	4.2	0

They are rank-ordered, from the highest to the lowest coefficients. Two relationships are shown for each enzyme, in an attempt to explain these differences in flux coefficients: The enzyme activity or V_{vivo} values for each enzyme, and the ratio of enzyme activity to reaction rate, which Atkinson discusses in the Prologue of this book. We find that this ratio is important, but clearly not the only critical variable which determines the magnitude of the enzyme flux coefficients.

Table 3 examines the effect of the V_{vivo}/flux ratio on these coefficients for one zero flux coefficient enzyme, fumarase, and one high coefficient enzyme, succinate dehydrogenase. When V_{vivo} was lowered in the first case, the substrate concentrations rose to unphysiological levels. Lowering the V_{vivo} value to approximate the reaction rate for this enzyme resulted in a change in the flux coefficients form zero to a significant value. Increasing the V_{vivo} value for a high coefficient enzyme, succinate dehydrogenase, decreased the substrate level and lowered the flux coefficient to zero. As might be anticipated, the ratio of V_{vivo}/flux also affects the sum of the coefficients of all enzymes on cycle flux. In the case of another zero coefficient enzyme, isocitrate dehydrogenase, using a V_{vivo} of 2.0, we did a complete

Table 4. Effect of V_{vivo} for isocitrate dehydrogenase on the sum of flux control coefficients

Affected reaction	Sum of control coefficients	
	High activity[1]	Low activity[2]
1	0.98	1.05
2	0.46	0.47
3	0.71	0.87
5	0.49	0.65
6	0.60	1.10
11	0.34	0.10
13	0.35	0.61

[1] V_{vivo} = 298 mM/min, [isocitrate] = 0.01 mM
[2] V_{vivo} = 2.0mM/min, [isocitrate] = 2.0 mM

Table 5. Effect of enzyme activity range on the sum of flux control coefficients

Affected reaction	Sum of control coefficients	
	Low range[1]	High range[2]
1	1.12	0.98
2	1.65	0.46
3	1.32	0.71
5	1.54	0.49
6	1.26	0.60
11	2.04	0.34
13	2.04	0.35

[1] Range from 30% to 100% of the model value
[2] Range from 50% to 200% of the model value

enzyme flux analysis for seven reactions, as shown in Table 4. The physiological model (V_{vivo} = 298) is shown for comparison. All but one value in the low V_{vivo} model are higher than in the physiological model, because reaction 13 (catalysed by isocitrate dehydrogenase) now contributes to the summed values. That is, the enzyme flux coefficient for this reaction has changed from zero to a positive value for most of the reactions.

As these data suggest, an important variable affecting enzyme flux coefficients is the range of enzyme activities chosen with which to calculate these coefficients. This is shown in Table 5. The coefficient for the effect of each enzyme on cycle flux was determined using two different ranges of enzyme activities: from 30% to 100% of the value in the model and from 50% to 200%. The sum of all the enzyme flux coefficients for seven cycle reactions is shown. As the V_{vivo} values all exceed the cycle flux, the lower range gives the higher coefficients. This range brings the V_{vivo} values closer to the cycle flux, and hence affects it more than the values used in the high range. Depending on the range used, the sum of the coefficients ("summation property") varies from 0.3 to 2.0.

It now becomes interesting to see which enzymes are most affected by the range chosen for calculating coefficients. In Table 6, four enzymes are compared. We examined one range below and two ranges above the model value. The effect of the range on the coefficient for the rate of the reaction catalysed is shown in the second column, and the sums of the flux control coefficients for reactions 13 and 3 are shown in the last two columns. These reactions were chosen as the simplest to represent flux through the cycle. The coefficients for aspartate transaminase are not affected by using different ranges, but the other enzyme coefficients are affected. In general, the higher the range, the lower the coefficient. In Fig. 2, the three ranges used for malic enzyme are shown graphically. The actual fluxes for the same three reactions are plotted as a function of V_{vivo} values for malic enzyme (flux 6). The effect on reaction 13, catalysed by isocitrate dehydrogenase, is interesting in that it is initially positive but becomes negative in the higher ranges.

Table 6. Effect of enzyme activity range flux control coefficients

Enzyme	Range of V_{vivo} (% of model value)	Flux control coefficient for own reaction	Sum of flux control coefficients Step 13	Step 3
Malate dehydrogenase	70 to 100	0.34	0.76	0.73
	100 to 130	0.26	0.67	0.73
	150 to 200	0.12	0.25	0.69
Malic enzyme	70 to 100	0.78	1.04	0.79
	100 to 130	0.63	0.41	0.73
	150 to 200	0.48	0.28	0.69
Aspartate transaminase	70 to 100	0.95	0.36	0.71
	100 to 130	0.94	0.34	0.71
	150 to 200	0.80	0.35	0.71
Succinate dehydrogenase	70 to 100	0.90	0.58	0.92
	100 to 130	0.73	0.44	0.75
	150 to 200	0.07	-0.09	0.09

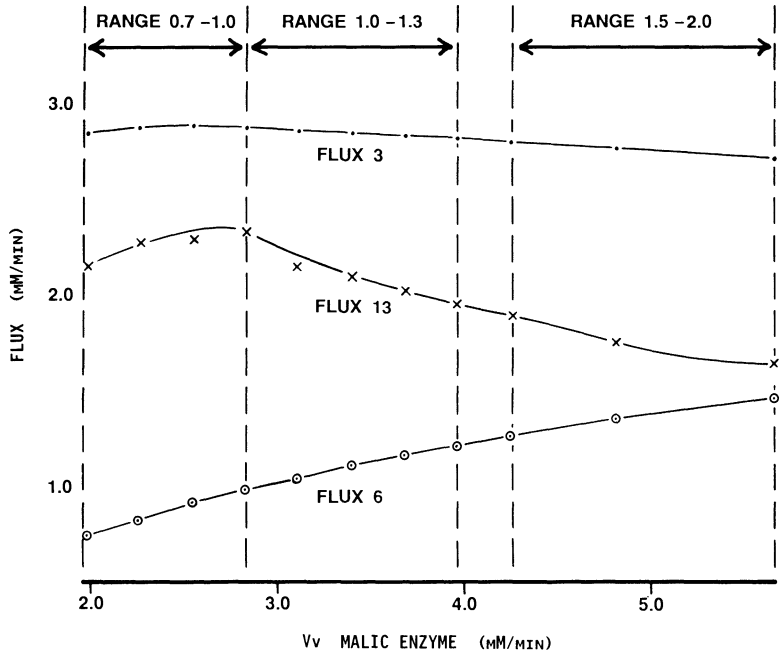


Figure 2. The fluxes for three reactions as a function of V_{vivo} values for malic enzyme (reaction 6).

In Fig. 3, the mechanisms underlying the range effect in the case of malic enzyme are illustrated. The low range values are shown in dashed line boxes and the high range is shown below them. The low range has less effect on the rate of the reaction; hence, less malate is used and malate levels are higher, while pyruvate and acetyl CoA levels are lower in the lower range than in the high range. The opposite is true for oxaloacetate, however, because malate levels are higher in the low range. As oxaloacetate is more rate-limiting than acetyl CoA, the rate of citrate synthesis is higher in the low range than it is in the high range. Consequently, the sum of the coefficients of enzymes on cycle flux are also higher in the low range. Thus, the rate of citrate synthesis actually undergoes a discontinuous change: it decreases in the high compared to the low range, because of the behaviour of the oxaloacetate pool.

In Table 7, we examine the compensatory effects of using different combinations of ranges for calculating coefficients for malic enzyme (reaction 6) and malate dehydrogenase (reaction 5). All possible combinations for these two enzymes were used and the sum of the resulting coefficients for seven reactions calculated. Note that some of the combinations give several values above unity, like the first two combinations; some give all values near unity, such as combinations 3 and 4; and some give several values that are quite low, like the last combination, with values of 0.17 and 0.18.

In Table 8, using two enzyme activity ranges, the sum of the coefficients of all enzymes on selected metabolites is shown. They clearly do not sum to zero. As with the sum of the

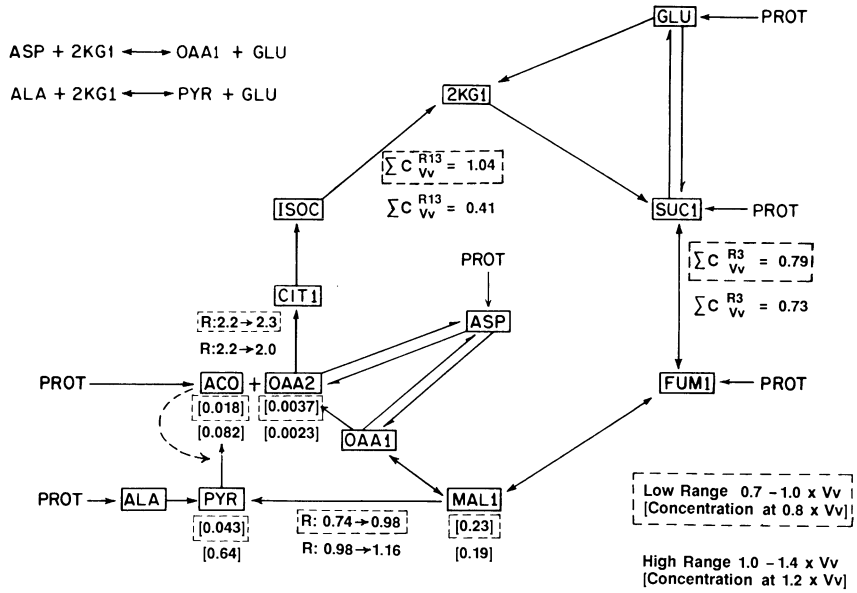


Figure 3. The effect of changes in the activity of malic enzyme on reaction rates and metabolite levels in the citric acid cycle. A low and a high range of enzyme activities were used.

coefficients of all enzymes on flux, the lowest range gives the highest coefficients.

As mentioned earlier, we wanted to be even-handed by also giving credit to metabolites in controlling flux and metabolite levels. As Krebs pointed out, metabolite availability controls cycle activity much more than enzyme activity. The mechanisms underlying metabolite effects are every bit as important as the mechanisms underlying enzyme effects. In Fig. 4, the role of one of the most interesting metabolites, malate, is analysed. Because of the mechanisms and kinetic constants for malate dehydrogenase and malic enzyme, higher

Table 7. Effect of ranges for malic enzyme and malate dehydrogenase on the sum of control coefficients for cycle flux

Malic enzyme	Malate dehydrogenase	Sum of flux control coefficients for reaction ...						
		1	2	3	5	6	11	13
70 to 100	70 to 100	0.96	1.50	0.81	0.92	0.96	1.44	1.45
	100 to 130	1.02	1.16	0.81	0.84	0.87	1.35	1.36
	150 to 200	0.96	1.15	0.77	0.70	0.77	0.93	0.94
100 to 130	70 to 100	0.99	0.86	0.75	0.73	0.81	0.81	0.82
	100 to 130	1.05	0.52	0.75	0.65	0.72	0.72	0.73
	150 to 200	0.99	0.51	0.71	0.51	0.62	0.30	0.31
150 to 200	70 to 100	0.97	0.75	0.71	0.54	0.66	0.68	0.69
	100 to 130	1.03	0.41	0.71	0.46	0.57	0.59	0.60
	150 to 200	0.97	0.40	0.67	0.32	0.47	0.17	0.18

Table 8. Effect of enzyme activity range on the sum of concentration control coefficients for individual metabolites, summed over all enzymes

Metabolite	30% to 100%	50% to 200%
Oxaloacetate	2.64	1.03
Acetyl CoA	3.51	-1.26
Pyruvate	3.70	-2.07
Glutamate	1.72	-0.03
Citrate	1.31	-0.62
2-Oxoglutarate	2.85	-1.59
Succinate	2.21	-1.31
Malate	0.98	-0.53

malate levels affect the rate of oxaloacetate synthesis somewhat more than the rate of pyruvate synthesis. The oxaloacetate levels increase from 0.0026 to 0.0031 mM (about 20%). As oxaloacetate is much more rate-limiting than acetyl-CoA in this bimolecular reaction, the rate of citrate synthesis increases: the coefficient is 0.69. This decreases the concentration of acetyl-CoA, which is an inhibitor of the pyruvate dehydrogenase complex. Hence, the rate of conversion of pyruvate to acetyl-CoA increases, resulting in decreased pyruvate levels, in spite of a positive coefficient for the rate of pyruvate formation from increased malate levels.

In summary, we found that V_{max} values for the well-characterized enzymes in this model could not be used, as they were incompatible with much more reliable data. Our results cast doubt upon the relevance of using *in vitro* enzyme activities for testing theories about the regulation of metabolism *in vivo*. It is highly unlikely that the relative activities of a series of enzymes in a cell extract is comparable to their relative activities *in vivo*.

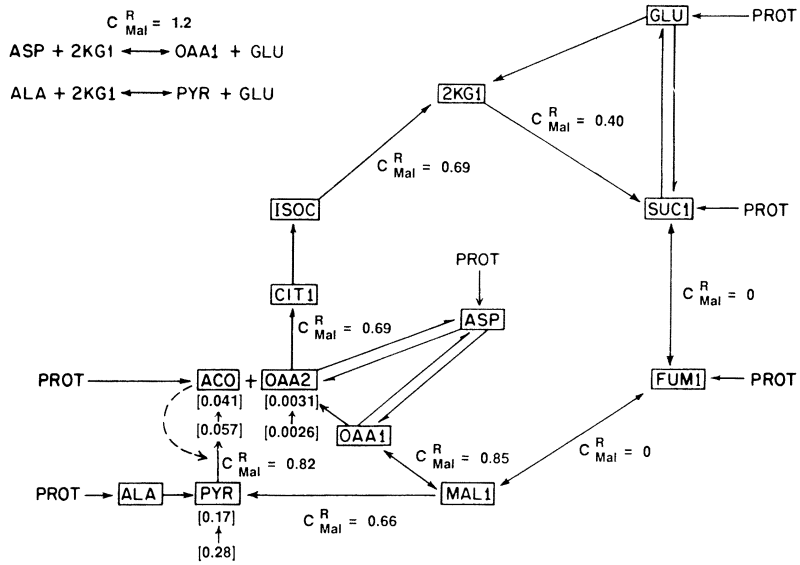


Figure 4. The effect of changes in the concentration of malate on reaction rates and metabolite levels in the citric acid cycle. The malate concentration was varied from 0.216 mM to 0.243 mM.

As V_{max} values were not a reliable indication of enzyme activity *in vivo*, we calculated this activity and called it V_{vivo} to distinguish it from V_{max}. Using V_{vivo} values, we calculated coefficients for the effect of each enzyme on the rate of its own reaction and found that the enzymes catalysing reactions 2, 4, 8, and 13 have zero or near zero coefficients; that is, these enzymes are not rate-limiting to cycle flux, and contribute nothing to flux control in this system. The rates of these reactions are controlled by substrate availability and end product inhibition. Enzymes with high flux coefficients catalyse reactions 1, 3, and 6. Changes in their activity have a strong effect on cycle flux. Critical variables affecting these coefficients and the summed coefficients on cycle flux are the ratio of V_{vivo}/rate and the range of enzyme activity values used to calculate the coefficients. Depending upon these variables, the sum of the coefficients of all enzymes on cycle flux varies from 0.2 to 2.0. The sum of the coefficients of all enzymes on each metabolite varies from -2.1 to 3.7. We therefore question the applicability of the "summation property" to realistically complex metabolic systems.

The results of this analysis raise a number of questions: Did we do it correctly from the theorist's point of view? If so, why don't their generalizations apply to our system? What experimental systems are valid for testing these theories? What questions do we want to ask? Coefficients *per se* are not very revealing. We believe most biochemists would be much more intrigued and enlightened by a series of "mechanism maps" describing in detail the reasons for the effects of each enzyme and each metabolite on the system. This is the level of mechanism which is exciting form our point of view. Exceptions to generalizations must not be suppressed or dismissed, as they hold the key to fascinating new insights into the ingenuity of living systems. Ockham has said that "Entities are not to be multiplied without necessity." However, when trying to understand the intricacies of metabolism *in vivo* it may well be necessary to multiply and integrate a great many entities. Clinging to oversimplified interpretations may interfere with a search for new relevant variables and delay our recognition of the complexity of the problem.

In conclusion, it is generally felt that enzymes must exert most of the control in metabolism because they contain so much information. However, it took other kinds of information to characterize these enzymes. Under the quasi-steady-state conditions of living cells, enzymes serve as part of a static structural framework — as catalysts to be manipulated by the environment. The dynamics of metabolic regulation at any point in time resides in the information inherent in the ever-changing patterns of available metabolites.

REFERENCES

Bloch, W., MacQuarrie, R. A. & Bernhard, S. A. (1971) *J. Biol. Chem. 246,* 780
Butler, M. H., Mell, G. P. & Wright, B. E. (1985) *Curr. Topics Cell. Reg. 26,* 337
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89.
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65
Kelleher, J. K., Kelly, P. J. & Wright, B. E. (1979) *J. Bacteriol. 138,* 467
Kelly, P. J. Kelleher, J. K. & Wright, B. E. (1979*a*) *Biochem. J. 184,* 581

Kelly, P. J. Kelleher, J. K. & Wright, B. E. (1979*b*) *Biochem. J. 184,* 589
Krebs, H. A. (1957) *Endeavour 16,* 125
Wistow, G. J., Mulders, J. W. M. & de Jong, W. W. (1987) *Nature 326,* 622
Wright, B. E. & Butler, M. H. (1987) in *Evolution of Longevity in Animals* (Woodhead, A. D. & Thompson, K. H., eds.) pp. 111-112, Plenum, New York
Wright, B. E. & Dahlberg, D. (1968) *J. Bacteriol. 95,* 983
Wright, B. E. & Kelly, P. J. (1981) *Curr. Topics Cell. Reg. 19,* 103.

ADDENDUM

On our return from the workshop recorded in this volume we explored the basis for our failure to obtain the (mathematically inevitable) summed flux coefficients of unity in the analysis of the citric acid cycle model. We believe we have now resolved the problem, and are indebted to a number of colleagues for helpful suggestions and offers of assistance. In particular we wish to thank Joe Higgins, David Fell, Athel Cornish-Bowden and Rafael Franco.

We had not anticipated how stringent the steady-state requirement is for being able to test the "summation properties", nor how small the perturbations must be. We established a "steady state" of less than 5% changes in any flux or metabolite level prior to our analysis of the citric acid cycle model. This seemed reasonable, as conditions *in vivo* are always quasi-steady-state, and in all likelihood such a large metabolic network is undergoing changes in excess of 5% over the time period involved. Perturbations of 10% also seemed reasonable, as experimental perturbations of this order are typically necessary to measure significant effects, and *in vitro* systems used to verify the flux "summation property" imply changes in flux and enzyme activitiues in excess of 10% [A. K. Groen *et al.* (1982), *J. Biol. Chem. 257,* 2754; N. V. Torres *et al.* (1986) *Biochem. J. 234,* 169; R. J. Middleton & H. Kacser (1983) *Genetics 105,* 633].

We have now examined two models which are in steady state to within 0.01%: the original 20-reaction model with six external inputs (Fig. 1) and a short linear series of three reactions (dead-end competitive inhibition, uni uni and mass action) with one external input to the first metabolite pool. After a 1% perturbation of the input flux to the latter model, 9 minutes were required to again reach steady state to within 0.01%; 30 minutes were required after a 6% perturbation: the sum of flux control coefficients was then 1.000. It was not possible to reach a new steady state after perturbation of the large 20-reaction model. Even a 0.01% change in the enzyme activity (V_{vivo}) of reaction 5 perturbed the system to the extent that a new steady state was not reached within 30 minutes. Longer time periods were not explored. Thus, using our METASIM program it is not theoretically possible to examine such a large metabolic network with respect to summed flux control coefficients.

We conclude that, even in a four-reaction system, a steady state with an accuracy to 0.01% must be achieved to test the flux control "summation property" and that perturbations must be of the order of 1% or less. We are puzzled about the implications of these conclusions, based on completely defined computer models, to the *in vitro* investigations referred to above. It is difficult to imagine that such precise steady state conditions could be established *in vitro,* or that such small perturbations could be made or measured accurately.

Chapter 28

Metabolic Control Analysis: Principles and Application to the Erythrocyte

REINHART HEINRICH

IT WAS PROPOSED (Heinrich & Rapoport, 1974*a*) to use as quantitative measures of metabolic control the following coefficients:

$$C_{v_j}^{J_k} = \frac{\partial \ln J_k}{\partial p} \Big/ \frac{\partial \ln v_j}{\partial p}; \qquad C_{v_j}^{S_i} = \frac{\partial \ln S_i}{\partial p} \Big/ \frac{\partial \ln v_j}{\partial p} \tag{1a,b}$$

where the notations are as follows: J_k, steady-state fluxes; S_i, steady-state concentrations; v_j, rates of isolated reactions; p, any kinetic parameter that affects primarily only one reaction j, i.e. $\partial v_k / \partial p = 0$ for $k \neq j$. It can be proved that the values of the control coefficients do not depend on the way in which the enzyme activities are changed, i.e. they are independent of the parameters that enter eqns. (1*a,b*).

If the reaction rates v_j are linearly dependent on the enzyme concentration, i.e. if $dv_j / v_j = dE_j / E_j$ and if there is no enzyme-enzyme interaction one may use the following definitions instead of eqns. (1*a,b*):

$$C_{v_j}^{J_k} = \frac{\partial \ln J_k}{\partial \ln E_j}; \qquad C_{v_j}^{S_i} = \frac{\partial \ln S_i}{\partial \ln E_j} \tag{2a,b}$$

(Kacser & Burns, 1973). The relative changes of steady state fluxes and steady-state metabolite concentrations after changes of system parameters may be characterized by response coefficients:

$$R_p^{J_k} = \frac{\partial \ln J_k}{\partial \ln p}; \qquad R_p^{S_i} = \frac{\partial \ln S_i}{\partial \ln p} \tag{3a,b}$$

Reinhart Heinrich ● Humboldt-Universität zu Berlin, Sektion Biologie, Bereich Biophysik, Invalidenstraße 42, 1040 Berlin, German Democratic Republic

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

$$P_1 \overset{v_1}{\Longleftrightarrow} S_1 \overset{v_2}{\Longleftrightarrow} S_2 \cdots S_{n-1} \overset{v_n}{\Longleftrightarrow} S_n \overset{v_{n+1}}{\Longleftrightarrow} P_2$$

Scheme 1. A simple linear pathway.

The response coefficient for the steady state flux ($R_p^{J_k}$) corresponds to the "control strength" defined by Higgins (1965). Control coefficients and response coefficients are related by the following formulas:

$$R_p^{J_k} = C_{v_j}^{J_k} \varepsilon_p^{v_j} \qquad R_p^{S_i} = C_{v_j}^{S_i} \varepsilon_p^{v_j} \tag{4a,b}$$

which contain the following elasticity coefficients:

$$\varepsilon_p^{v_j} = \frac{\partial \ln v_j}{\partial \ln p} \tag{5}$$

The possibility to factorize the total response into systemic coefficients ($C_{v_j}^{J_k}$, $C_{v_j}^{S_i}$) that are independent of the special choice of the perturbation parameter and a term ($\varepsilon_p^{v_j}$) depending on the kinetic parameters of only one enzyme is the reason why it is useful to consider control coefficients and elasticity coefficients separately.

A Simple Example: Control of Linear Enzymatic Chains

The dynamic behaviour of a linear enzymatic pathway (Scheme 1) is governed by the following differential equations:

$$\frac{dS_i}{dt} = v_i - v_{i+1} \tag{6}$$

with the side conditions $S_0 = P_1 = \text{constant}$, $S_{n+1} = P_2 = \text{constant}$. If all reactions follow the reversible form of the Michaelis-Menten equation:

$$v_i = \frac{(V_i^+/K_i^+)S_{i-1} - (V_i^-/K_i^-)S_i}{1 + S_{i-1}/K_i^+ + S_i/K_i^-} \tag{7}$$

one gets under steady state conditions ($dS_i/dt = 0$, $v_i = J$) the following nonlinear equation for the steady state flux J:

$$p_2 = p_1 \prod_{j=1}^{i} \frac{K_j^-(V_j^+ - J)}{K_j^+(V_j^- + J)} - J \sum_{l=1}^{i} \frac{K_l}{V_l^+ - J} \prod_{j=l}^{i} \frac{K_j^-(V_j^+ - J)}{K_j^+(V_j^- + J)} \tag{8}$$

This may be rearranged into a polynomial equation for J, the degree of this equation corresponding to the number of reactions within the chain.

To arrive at explicit solutions, one may introduce the approximation that for all enzymes the concentrations of substrates and products are well below the corresponding Michaelis constants:

$$v_i = k_i S_{i-1} - k_{-i} S_i = k_i (S_{i-1} - S_i/q_i) \tag{9}$$

with $k_i = V_i^+/K_i^+$, $k_{-i} = V_i^-/K_i^-$, $q_i = k_i/k_{-i}$. Using eqn. (6) the following formulas are derived for the steady-state flux and for the metabolite concentrations:

$$J = \frac{p_1 \prod_{j=1}^{n+1} q_j - p_2}{\sum_{l=1}^{n+1} \frac{1}{k_l} \prod_{j=l+1}^{n+1} q_j}; \qquad S_i = p_1 \prod_{j=1}^{n+1} q_j - J \sum_{l=1}^{n+1} \frac{1}{k_l} \prod_{j=l+1}^{n+1} q_j \tag{10a,b}$$

For the control coefficients, the following formulas are obtained:

$$C_{v_j}^J = \frac{\tau_j (1+q_j) \prod_{i=j+1}^{n+1} q_i}{\sum_{k=1}^{n+1} \tau_k (1+q_k) \prod_{i=k+1}^{n+1} q_i}; \qquad C_{v_j}^{S_i} = \begin{cases} C_{v_j}^J & \text{for } j \le i \\ -C_{v_j}^J \sum_{k=1}^{i} C_{v_k}^J \Big/ \left(1 - \sum_{k=1}^{i} C_{v_k}^J\right) & \text{for } j > i \end{cases} \tag{11a,b}$$

with the characteristic times $\tau_i = 1/(k_i + k_{-i})$. An inspection of eqns. (11a,b) shows that the control coefficients of very fast enzymes (with short relaxation times) become negligibly small. However, the control coefficients do not only depend on the characteristic times but also on the equilibrium constants as well as on the position of the enzyme within the chain. Only for the case that all the equilibrium constants are equal to one, the distribution of control coefficients corresponds to the distribution of the characteristic times. In another case, where the equilibrium constant of one enzyme is much greater than of all others the control coefficients of reactions behind the quasi-irreversible step become negligible small.

From eqns. (11*a,b*), it immediately follows that the sum of flux control coefficients is equal to one while for a given metabolite the sum of the concentration control coefficients is equal to zero. It could be shown that these summation relationships are generally valid irrespective of the stoicheiometry of the network and of the linear or nonlinear character of the kinetic equations (cf. below).

Calculation of Control Coefficients and Theorems of Control Analysis

Control coefficients may be calculated on the basis of elasticity coefficients. This may be shown by implicit differentiation of the system equations:

$$\frac{dS_i}{dt} = \sum_{j=1}^{r} a_{ij} v_j; \quad i = 1 \cdots n \tag{12}$$

with respect to a system parameter p which affects primarily only the rate of one reaction v_m (Heinrich & Rapoport, 1974b, 1975). Notations in eqn. (12) are as follows: r, number of reactions; n, number of metabolites; a_{ij}, stoicheiometric coefficients. From eqn. (12) one gets the following under steady-state conditions ($dS_i/dt = 0$):

$$\sum_{j=1}^{r}\sum_{k=1}^{n} a_{ij}\frac{\partial v_j \partial S_k}{\partial S_k \partial p} + a_{im}\frac{\partial v_m}{\partial p} = 0 \tag{13}$$

Using the following abbreviation,

$$m_{ik} = \sum_{j=1}^{r} a_{ij}\frac{\partial v_j}{\partial S_k} = \sum_{j=1}^{r} a_{ij}\frac{v_j}{S_k}\varepsilon_{S_k}^{v_j} \tag{14}$$

where m_{ik} are the elements of the Jacobi-matrix **M**, eqn. (13) may be written as follows:

$$\sum_{k=1}^{n} m_{ik}\frac{\partial S_k}{\partial p} = -a_{im}\frac{\partial v_m}{\partial p} \tag{15}$$

If the Jacobi-matrix is regular, i.e. the system does not contain conservation quantities and the solution of eqn. (12) with respect to the steady state concentrations is isolated, one obtains the following from eqn. (15):

$$C_{v_j}^{S_i} = \frac{v_j \partial S_i/\partial p}{S_i \partial v_j/\partial p} = -\sum_{k=1}^{n} m_{ik}^{-1} a_{kj}\frac{v_j}{S_i} \tag{16}$$

where m_{ik}^{-1} are the elements of the inverse of the Jacobi-matrix. Using eqns. (15-16) for the calculation of the concentration control coefficients one must know besides of the elasticity coefficients the fluxes and the metabolite concentrations within the reference state.

The flux control coefficients may be derived from the concentration control coefficients using the following formula:

$$C_{v_j}^{J_k} = \delta_{ki} + \sum_{i=1}^{n} \varepsilon_{S_i}^{v_j} C_{v_j}^{S_i} \tag{17}$$

(Heinrich & Rapoport, 1974*b*, 1975). The calculation method described above was generalized by consideration of systems containing conservation quantities (Reder, 1986, 1988).

Using eqns. (12) and (14), one obtains from eqns. (16) and (17) by summation

$$\sum_{j=1}^{r} C_{v_j}^{S_i} = 0; \qquad \sum_{j=1}^{r} C_{v_j}^{J_k} = 1 \tag{18*a,b*}$$

(Summation theorems: Kacser & Burns, 1973; Heinrich & Rapoport, 1974*a*). Multiplication of (16) with the elasticity coefficient yields the following after summation, taking eqn. (14) into account:

$$\sum_{j=1}^{r} C_{v_j}^{J_k}\varepsilon_{S_i}^{v_j} = 0; \qquad \sum_{j=1}^{r} C_{v_j}^{S_k}\varepsilon_{S_i}^{v_j} = -\delta_{ki} \tag{19*a,b*}$$

(Connectivity theorems: Kacser & Burns, 1973; Westerhoff & Chen, 1984). Recently, Reder (1986, 1988) showed that eqns. (19*a,b*) are special cases of generalized summation theorems.

Formulas (12-19) show that the validity of the theorems of control analysis is not restricted to cases where the reaction rates are proportional to the enzyme concentrations. This was until recently a controversial point [cf. Discussion Forum in *Trends Biochem. Sci. 12,* 216-224 (1987)]. There is not even any need that all reactions are catalysed by enzymes. The theorems are applicable to all systems that can be described by the set of eqns. (12) provided the general definitions (1*a,b*) of control coefficients are used.

Time-dependent control coefficients. Usually, control analysis is applied to characterize the changes of system variables after perturbation of system parameters for $t \to \infty$, i.e. for full relaxation of the system. However, very often the transition to the true steady state cannot be observed due to the slowness of some reactions. It is, therefore, worthwhile to define time-dependent control coefficients that characterize the response of the system variables for finite time intervals after a small parameter perturbation. With $p \to p + \delta p$ at $t = 0$ ($\partial v_k/\partial p = 0$ for $k \neq m$), and $S_i \to S_i + \delta S_i(t)$, where p and S_i are the parameter value and steady-state concentrations, respectively, for $t < 0$) one gets the following result from the system equations, eqns. (12), in a linear approximation:

$$\frac{\mathrm{d}}{\mathrm{d}t}[\delta S_i(t)] = \sum_{j=1}^{r} \sum_{k=1}^{n} a_{ij} \frac{\partial v_j}{\partial S_k} \delta S_k(t) + a_{im} \frac{\partial v_m}{\partial p} \delta p \tag{20}$$

Using the definition

$$\frac{v_m}{S_i}\left[\frac{\delta S_i(t)/\delta p}{\partial v_m/\partial p}\right]_{\delta p \to 0} = C^{S_i}_{v_m}(t) \tag{21}$$

eqn. (20) may be rewritten as follows:

$$\frac{\mathrm{d}}{\mathrm{d}t} C^{S_i}_{v_m}(t) = \sum_{k=1}^{n} m_{ik} \frac{S_k}{S_i} C^{S_k}_{v_m} + a_{im} \frac{v_m}{S_i} \tag{22}$$

with m_{ik} defined as in eqn. (14), (cf. Heinrich, 1985). The time-independent solution of eqn. (22) yields the usual control coefficients given in formula (16). For finite times eqn. (22) may be solved by standard techniques for linear differential equation systems. Since $C^{S_i}_{v_m}(t=0) = 0$ one obtains by summation of eqn. (22) with respect to index m the result that also the time-dependent control coefficients fulfil the summation theorem (18*a*) for all times $t > 0$ (provided the steady state considered is stable).

Eqn. (22) may also be used to derive a connectivity theorem for time dependent control coefficients. One obtains

$$\sum_{j=1}^{r} C^{S_k}_{v_j}(t) \varepsilon^{v_j}_{S_i} = \frac{S_k}{S_i} \sum_{m=1}^{n} b_{km}(e^{\lambda_m t} - 1) b^{-1}_{mi} \tag{23}$$

in which λ_m are the eigenvalues of the Jacobi-matrix and the columns of the matrix $\mathbf{B} = \{b_{km}\}$ are the corresponding eigenvectors; b^{-1}_{mi} are the elements of the matrix $\mathbf{B}^{-1}$. If the steady state is stable (Re $\lambda_m < 0$ for all m) the right hand side converges for $t \to \infty$, i.e. one

obtains the usual connectivity theorem, eqn. (19*b*). Eqns. (22-23) together with similar equations for time-dependent flux control coefficients may be useful for the determination of control coefficients in quasi-steady states.

Mathematical Models of Erythrocyte Metabolism

Early models of erythrocyte metabolism (Rapoport *et al.*, 1974, 1976; Heinrich *et al.*, 1977) were based on reaction schemes that include the glycolytic enzymes transforming glucose to lactate with a net production of ATP. Furthermore, non-glycolytic ATP-consuming processes were taken into account as well as the 2,3-bisphosphoglycerate bypass, which is typical for erythrocytes. The glycolytic pathway includes slow and very fast reaction steps. This separation of time constants (time hierarchy) allowed the application of the quasi-steady-state approximation, leading to simplifications of the model as described below. For models of erythrocyte glycolysis the steady-state solutions show the following properties:

1. There exists a critical parameter value (bifurcation point) for the kinetic constant of non-glycolytic ATP-consuming processes k_{ATPase}. For k_{ATPase} greater than a critical value there is only the trivial solution with an ATP concentration of zero and glycolytic flux of zero.

2. There exists a certain region for the parameter k_{ATPase} where unstable steady states exist.

3. For low values of k_{ATPase} the ATP concentration is rather insensitive to variations of the kinetic constants of ATP-consuming processes. These properties of the steady state solutions were found also in skeleton models of glycolysis (Selkov, 1975). The model of erythrocyte metabolism has recently been extended by incorporation of the pentose-phosphate pathway (Schuster *et al.*, 1988).

Fig. 1 shows the reaction scheme of a metabolic-osmotic model of erythrocyte energy metabolism (Brumen & Heinrich, 1984); Werner & Heinrich, 1985). The previous models were extended by inclusion of transmembrane fluxes of sodium, potassium, chloride and lactate. The model contains two different non-glycolytic ATP-consuming processes, the Na/K-ATPase and a non-ion-transport ATPase. Two situations are considered: (1) the "open system" with fixed composition of the external medium, in which the intracellular variables may attain a stationary *in vivo* state, and (2) the "closed system" with a finite extracellular volume (*in vitro* state).

In this metabolic osmotic model the set of system variables contains the cellular volume (V_i) and the transmembrane potential ($\Delta\Psi$). The set of parameters is enlarged by quantities characterizing the osmotic action of haemoglobin and the electric charges of haemoglobin and other compounds. Further system parameters are the permeabilities of ions.

The system equations for this model consist of a coupled set of non-linear parameter dependent differential equations and algebraic constraints (algebro-differential equations).

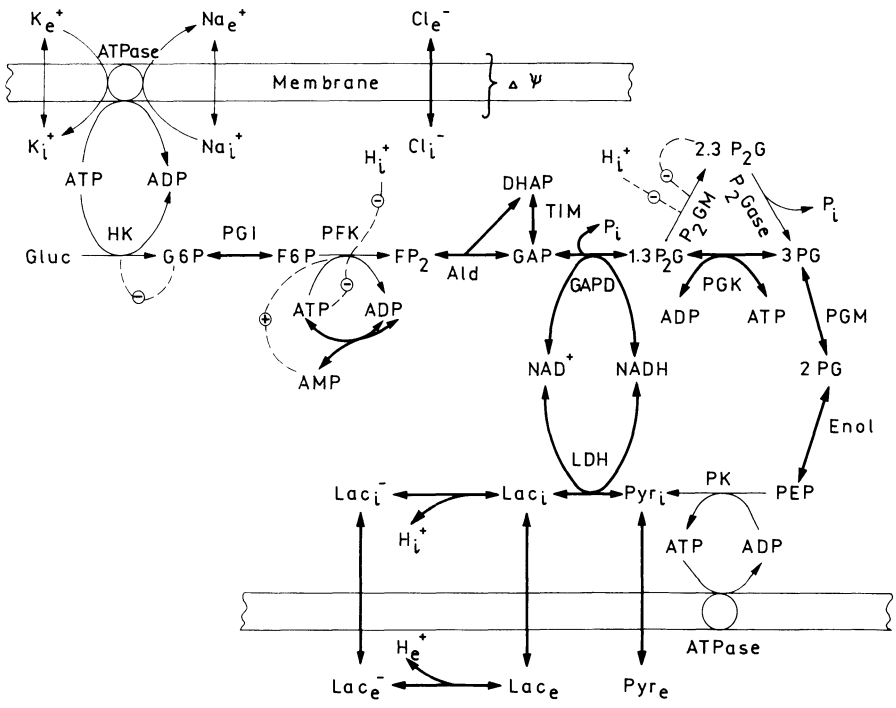


Figure 1. Reaction scheme for the metabolic osmotic model of human erythrocytes.

The differential equations for the concentrations (S_j) of the glycolytic intermediates are derived from the stoicheiometry of the system and the kinetic equations of the reactions:

$$\frac{1}{V_i^0}\frac{d}{dt}(S_j V_i) = \sum_k a_{jk} v_k \tag{24}$$

in which V_i^0 is the cellular volume in the reference state. This equation takes into account that the concentrations are changed not only by the reactions (v_k) but also by variations of the cell volume (V_i). For the reaction rates v_k nonlinear functions derived from the kinetic properties of the isolated enzymes are used.

Differential equations are also used to describe the transmembrane transport of sodium and potassium. Taking into account that Na/K-ATPase carries 3 mol sodium outwards and 2 mol potassium inwards mol ATP degraded the time-dependent changes of the cellular cation concentration are governed by the following differential equations:

$$\frac{1}{V_i^0}\frac{d}{dt}(Na_i^+ V_i) = v_{Na,\,passive} - 3v_{Na/K\text{-}ATPase} \tag{25a}$$

$$\frac{1}{V_i^0}\frac{d}{dt}(K_i^+ V_i) = v_{K,\,passive} + 2v_{Na/K\text{-}ATPase} \tag{25b}$$

The passive fluxes depend on the transmembrane potential. In the present model they are described by Goldman's flux equation (Goldman, 1943). Application of the quasi-steady-state approximation to the fast transport processes of the anions chloride and lactate leads to equilibrium distributions for internal and external concentrations:

$$Cl_e^- = Cl_i^-/r; \qquad Lactate_e^- = Lactate_i^-/r \tag{26}$$

in which $r = \exp(F\Delta\Psi/RT$. The equilibrium assumption is also applied to calculate the relationship between intra- and extracellular pH:

$$pH_e = pH_i - \log r \tag{27}$$

Under *in vitro* conditions, the total volume consisting of the cellular volume (V_i) and the plasma volume (V_e) is time-independent. A conservation equation for the volume is used to express the extracellular volume as a function of the intracellular volume. Other conservation equations result from the fact that under *in vitro* conditions the total contents of the permeable ions chloride, sodium and potassium within the cell and the extracellular compartment are constant. For any substance X this conservation equation reads as follows:

$$X_i^0 V_i^0 + X_e^0 V_e^0 = X_i(t)V_i(t) = X_e(t)V_e(t) \tag{28}$$

where X_i^0 and X_e^0 denote respectively the internal and external concentrations under standard conditions. Further algebraic constraints are obtained from the electroneutrality conditions for the intra- and extracellular compartments as well as from the condition of osmotic equilibrium of the cell with the surrounding medium (for details see Werner & Heinrich, 1985).

The steady state solution of eqns. (24-28) constitutes the reference state of the model (*in vivo* state, $V_e \to \infty$). It was used as the starting point for the calculation of time-dependent states. It was proved that the *in vivo* state is always stable. In contrast to the energy metabolism of some other cells, glycolysis of erythrocytes does not show self-sustained oscillations (limit cycles). This prediction is confirmed by experimental data.

The model equations were used to calculate time-dependent variations of all system variables under blood preservation conditions (for 37°C). Here, a stationary state cannot be maintained as the cell and the surrounding plasma constitute a closed system. The accumulation of lactate is accompanied by a lowering of the extracellular and intracellular pH. Since phosphofructokinase is inhibited by hydrogen ions, drastic influences on the metabolic state are observed. The decrease of the ATP concentration affects the transport processes and the osmotic state of erythrocytes. The results presented in Figs. 2-4 were obtained by numerical integration (cf. Werner & Heinrich, 1985). Under blood preservation conditions the glycolytic rate is strongly diminished which leads to a breakdown of the concentrations of 2,3-bisphosphoglycerate, ATP and other glycolytic compounds (Fig. 2). Due to the lowering of the ATP concentration the ATP-consuming Na/K-ATPase can no longer maintain the concentration gradient of K^+ and Na^+ across the cellular membrane. The concentrations of these ions undergo a transition to an equilibrium-state (Donnan-state) that is characterized by a passive distribution of all permeable ions (Fig. 3). The redistribution of ions and the changes of the

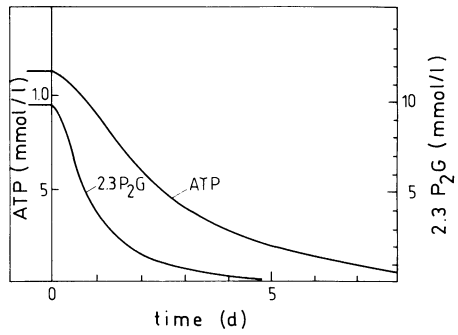


Figure 2. Breakdown of the concentration of ATP and 2,3-bisphosphoglycerate ($2,3P_2G$) under blood preservation conditions.

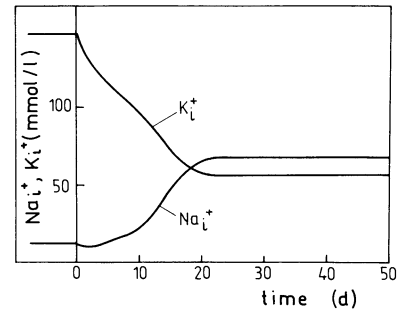


Figure 3. Time-dependent changes of intracellular K^+ and Na^+ concentrations under blood preservation conditions.

pH-dependent charges of haemoglobin and 2,3-bisphosphoglycerate, lead to strong variations of the intracellular pH and of the transmembrane potential (Fig. 4). The transmembrane potential changes from -9 mV *in vivo* state to about 9 mV in the Donnan-state (cf. Brumen *et al.*, 1979; Glaser *et al.*, 1980).

Control Coefficients of Erythrocyte Glycolysis

On the basis of the system equations of the metabolic-osmotic model control coefficients for the *in vivo* stage were calculated. Table 1 lists the control coefficients for the glycolytic rate and for the ATP concentration. Furthermore, the model allows the calculation of control coefficients for the cellular volume (last column in Table 1). Obviously, in this metabolic osmotic model the system variables are not only affected by the activity of the enzymes but also by the rates of active and passive transport processes. The corresponding control coefficients are included in Table 1.

As for other complex systems, positive as well as negative control coefficients are obtained. Among all the enzymes considered in the model the activity of the hexokinase/

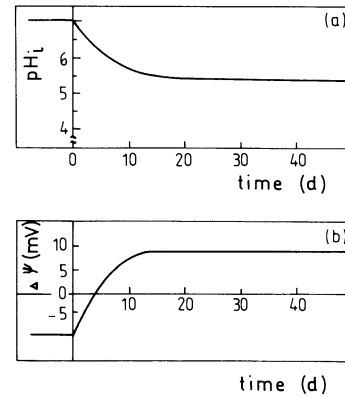


Figure 4. Time-dependent changes of the intracellular pH (*a*) and of the transmembrane potential (*b*) under blood preservation conditions.

Table 1. Control coefficients of the metabolic osmotic model of human erythrocytes for glycolytic flux (J), ATP concentration and internal volume (V) for the "*in vivo*" steady state

Reaction (v_j)	$C^J_{v_j}$	$C^{ATP}_{v_j}$	$C^V_{v_j}$
Hexokinase + phosphofructokinase	1.13	-0.09	0.68
Bisphosphoglycerate mutase	0.31	-0.22	0.19
Bisphosphoglycerate phosphatase	-0.30	0.24	-0.39
Phosphoglycerate kinase	-0.31	0.22	-0.19
Na/K ATPase	-0.05	0.03	-0.06
ATPase	0.16	-0.11	-0.21
Passive K^+ transport	-4.19	2.93	-4.83
Passive Na^+ transport	4.30	-3.00	4.81
Sums	1.00	0.00	0.00

phosphofructokinase system exerts the most significant control on the glycolytic flux. This corresponds to results obtained in earlier models of erythrocyte metabolism (Rapoport *et al.*, 1974, 1976; Heinrich *et al.*, 1977). The hexokinase/phosphofructokinase system has a high control coefficient also for the cellular volume. The control coefficients of glycolytic enzymes for the ATP-concentration are rather small. This homeostasis of the steady-state level of the ATP-concentration results from regulatory couplings, mainly from the ATP-inhibition of the phosphofructokinase, but also from the 2,3-bisphosphoglycerate bypass, which is switched off at increasing ATP-consumption. The separate control effects of the cation leak permeabilities are enormous. They are obviously exerted by affecting the cell cation content which is followed by corresponding changes of the cellular volume and the metabolite concentrations. The simultaneous control of both permeabilities is very small since their control coefficients are of opposite sign. A detailed discussion of the control coefficients for the metabolic osmotic model of erythrocytes may be found in Brumen & Heinrich (1984).

Simplification of Metabolic Models

Metabolic pathways contain a high number of interrelated reactions with complicated kinetic properties. Realistic models of such systems consist, therefore, of many differential equations. They depend in a nonlinear manner on the variables and parameters that prevents analytic solutions. One may ask whether it is possible to simplify the mathematical treatment.

Very often a model simplification can be achieved by application of the quasi-steady state approximation which is mathematically based on Tikhonov's theorem (Tikhonov, 1948). In many cases it may be applied in the following way. Let us consider the case that the vector $\mathbf{v} = (v_1 \ldots v_r)^T$ of the reaction rates may be subdivided into two vectors $\mathbf{u} = (u_1 \ldots u_r)^T$ and $\mathbf{w} = (w_1 \ldots w_r)^T$ containing the rates of slow and very fast reversible reactions, respectively. This implies a subdivision of the matrix $\mathbf{A} = \{a_{ij}\}$ into the stoicheiometric matrices $\mathbf{A}^{(1)} = \{a^{(1)}_{ij}\}$ and $\mathbf{A}^{(2)} = \{a^{(2)}_{ij}\}$ of the slow and fast subsystems, respectively. Then the system equations may be reformulated as follows:

$$\frac{dS_i}{dt} = \sum_{j=1}^{\rho} a^{(1)}_{ij} u_j + \frac{1}{\mu} \sum_{j=\rho+1}^{r} a^{(2)}_{ij} \tilde{w}_j \qquad (29)$$

In eqn. (29) the rates of the fast reactions are normalized by a small parameter μ such that $\tilde{\mathbf{w}} = \mu \cdot \mathbf{w}$. Before going to the limit $\mu \to 0$ it is necessary to derive from eqn. (29) a maximal number of differential equations that do not contain the small parameter μ. For that one may introduce pool variables $\mathbf{Y} = (Y_1 \ldots Y_\nu)^T$ that are linear combinations of the original variables $\mathbf{S} = (S_1 \ldots S_n)^T$

$$Y_i = \sum_{j=1}^{n} t_{ij} S_j; \quad i = 1 \cdots \nu \tag{30}$$

The transformation matrix $\mathbf{T} = \{t_{ij}\}$ must fulfil the following condition:

$$\sum_{j=1}^{n} t_{ij} a_{ij}^{(2)} = 0 \tag{31}$$

According to eqns. (29-31) the slow pool variables represent the conservation quantities of the fast subsystem. Their number (ν) depends on the rank of the stoicheiometric matrix $\mathbf{A}^{(2)}$ ($\nu = n - a_2$; a_2 = rank $\mathbf{A}^{(2)}$; for details see Schauer & Heinrich, 1983; Schuster & Schuster, 1989). In the limit $\mu \to 0$ one has to solve instead of the original differential eqns. (29) the following system of algebro-differential equations:

$$\frac{dY_i}{dt} = \sum_{j=1}^{n} \sum_{k=1}^{\zeta} t_{ij} a_{jk}^{(1)} u_k; \quad i = 1 \cdots \nu \tag{32a}$$

$$0 = \sum_{k=\rho+1}^{r} a_{ik}^{(2)} \tilde{\omega}_k; \quad i = 1 \cdots n \tag{32b}$$

For the calculation of the transformation matrix T a general method was proposed (Schauer & Heinrich, 1983).

If for the fast subsystem a generalized "Wegscheider condition" is fulfilled (Schuster & Schuster, 1989) eqns. (32*b*) imply that the fast reversible reactions are in equilibrium, i.e.

$$\tilde{\omega}_k = 0; \quad k = (\rho+1) \cdots r \tag{33}$$

In many cases the algebro-differential eqns. (32*ab*) are much easier to solve than the original system, eqn. (29). This holds, in particular, if eqn. (33) can be applied, i.e. if in a long time scale all fast reactions may be considered to be in equilibrium. In such a case only the equilibrium constants of these reactions enter the model (reduction of the number of parameters). Furthermore, the speed of the integration is enhanced since the remaining differential eqns. (32*a*) for the slow pool variables are less stiff than the original eqns. (29).

Theoretical Analysis of the Structural Design of Metabolic Pathways

In contrast to chemical systems the biochemical reaction networks are the result of natural selection where the contemporary metabolic systems have been developed by a stepwise improvement of the functioning of the different subsystems of the cell. Certainly, this development did not lead to a "global optimal state". However, it is an experimental fact that

a change of the kinetic parameters of enzymes by mutations in contemporary metabolic systems mostly results in a worse biological function. From this, one may conclude that with respect to present-day metabolic systems, we are confronted at least with a "local optimum". It seems, therefore, to be a meaningful task to explain the structural design of metabolic networks on the basis of optimality principles. Such a theoretical analysis may be considered to be complementary to the classical approach of modelling. Simulation models explored in biochemistry over more then 20 years cannot provide rules for understanding the functional organization of metabolic systems, i.e. questions as: "Why has the Michaelis-constant of a given enzyme just this value rather than another?"; "Why is this enzyme allosterically inhibited by that end product?"; "Why is this enzyme much slower than another?" or "Why do we find this and not another distribution of control coefficients?" remain unanswered.

It was proposed to calculate kinetic parameters of metabolic pathways on the basis of the assumption that during evolution some performance function Φ was optimized (Waley, 1964; Chernavskij *et al.*, 1976; Reich, 1984, 1985; Heinrich & Holzhütter, 1985; Heinrich *et al.*, 1987; see also Chapter 10 of this book, by Cascante, Franco and Canela, which is devoted to consideration of performance indices). Obviously, any performance function should depend on the physical properties characterizing metabolic systems, i.e. on the metabolite concentrations and the fluxes. On the basis of the system equations, the variables S_1 and J_k may be expressed by the kinetic parameters. One may write, therefore,

$$\phi = \phi(\mathbf{S}, \mathbf{J}) = \phi^*(\mathbf{p}) \tag{34}$$

The crucial point is to identify systemic properties that may serve as relevant performance functions. Metabolic networks represent open nonequilibrium systems and no cell could survive if there would be only vanishing fluxes. Therefore, it may be assumed that metabolic fluxes were targets of optimization during evolution. Of course, many other performance function could be of importance (see below). Naturally, any optimization has to be performed under consideration of side conditions $\chi_i = \chi_i(\mathbf{S}, \mathbf{J}, \mathbf{p}) = \chi^*_i(\mathbf{p}) \leq \chi_i$. For example, the total concentration of enzymes within a cell cannot exceed an upper limit. This holds true also for the total concentration of internal metabolites due to the limited solvent capacity of the cell (Atkinson, 1969). Under certain conditions such constraints can be taken into account using the method of Lagrange multipliers (Heinrich & Holzhütter, 1985; Heinrich *et al.*, 1987).

Let us consider here the most simple example, the optimization of the flux through a linear enzymatic chain. The starting point is eqn. (10*a*) for the steady-state flux as a function of the kinetic constants k_1 and the equilibrium constants q_1. If this expression for the flux is used as performance function ($\Phi = J$) and if it is maximized under the side condition that the total concentration of enzymes cannot exceed an upper limit one arrives at the following variational equation for the optimal distribution of the first order kinetic constants:

$$\frac{\partial J}{\partial k_i} + \lambda \frac{\partial}{\partial k_i} \sum_{j=1}^{n+1} \frac{k_j}{\alpha_j} = 0; \qquad \sum_{j=1}^{n+1} E_j = \sum_{j=1}^{n+1} \frac{k_j}{\alpha_j} = \chi^0 \tag{35}$$

where λ is a Lagrange multiplier. For $\alpha_j = \alpha$ one gets from consideration of eqn. (10*a*) the following distribution of kinetic parameters:

$$\tilde{k}_i = (\chi^0 \alpha) \frac{\prod_{j=1}^{i-1} (1/q_j)^{1/2}}{\sum_{k=1}^{n+1} \prod_{j=1}^{k-1} (1/q_j)^{1/2}} \tag{36}$$

The optimal values $\tilde{k}_i$ depend on the equilibrium constants, which must be considered as fixed quantities during evolution. For the case that all equilibrium constants are greater than one eqn. (36) predicts a decrease of kinetic constants toward the end of the chain.

Optimal states of linear enzymatic chains were calculated also under more general assumptions using nonlinear kinetic equations for the individual reaction steps and by consideration of constraints for the total concentrations of intermediates (Heinrich *et al.*, 1987). Using the optimal parameter values one may calculate also the corresponding distribution of control coefficients. In the simplest case where the steady state flux is maximized under the side condition of a fixed sum of enzyme concentrations the variational eqn. (35) leads (for $\alpha_j = \alpha, j = 1 \ldots n + 1$) directly to the result that within the optimal state the distribution of control coefficients corresponds to the distribution of the kinetic constants:

$$C_{v}^{J} / C_{v_j}^{J} = \tilde{k}_i / \tilde{k}_j \tag{37}$$

Eqn. (37) reflects a rather surprising result. For $q_j > 1$ ($j = 1 \ldots n + 1$) the fast enzymes at the beginning of the chain and not the slow enzymes at the end have high flux control coefficients. This conclusion underlines the fact that the values of control coefficients are strongly dependent on the position of the enzyme within the metabolic network.

The optimization method may be extended to branched metabolic pathways with more than one steady state flux. One may identify generally $r - a$ different steady state fluxes, where r is the total number of reactions and a is the rank of the stoicheiometric matrix **A**). As long as the stoicheiometry of the pathway is considered to be fixed only $r - a$ steady-state fluxes can be independently optimized. It may be argued, therefore, that the number $r - a$ is closely related to the number of different biological functions that are fulfilled by the metabolic network.

Optimization of fluxes does not lead to a strong separation of time constants. However, separated time constants are typically for all metabolic systems. In glycolysis of erythrocytes, for example, the relaxation times of the reactions differ by more than four orders of magnitude. The slowest processes have relaxation times of several hours and there are many reactions with relaxation times much shorter than one second. The evolutionary meaning of such a strong separation of time constants is still unclear. Probably, time hierarchy is related to the stability of metabolic networks. The hypothesis was formulated that evolution toward systems with separated time constants allowed simple dynamic behaviour despite increasing structural complexity (Heinrich *et al.*, 1977; Heinrich & Sonntag, 1982). This hypothesis is supported by results of recent calculations using optimization procedures (Schuster &

Heinrich, 1987). As performance functions the real parts of the eigenvalues of the Jacobi-matrix were used. Minimization of the largest eigenvalue (i.e. optimization of stability) under the side condition of fixed steady state fluxes and fixed total concentrations of intermediates yields optimal parameter distributions showing strong separation of time constants. In the optimal state there are always a small number of slow reactions with high control coefficients and a great number of very fast reactions that exert no control.

REFERENCES

Atkinson, D. E. (1969) *Curr. Topics Cell. Regul. 1,* 29
Brumen, M., Glaser, R. & Svetina, S. (1979) *Bioelectrochem. and Bioenergetics 6,* 227
Brumen, M. & Heinrich, R. (1984) *BioSystems 17,* 155
Chernavskij, D. S., Volkov, E. I. & Presnov, E. V. (1976) *Biofizika 21*, 334
Glaser, R., Brumen, M. & Svetina S. (1980) *Biol. Zentralbl. 99,* 429
Goldman, D. E. (1943) *J. Gen. Physiol. 27,* 37
Heinrich, R. (1985) *Biomed. Biochim. Acta 44,* 913
Heinrich, R. & Holzhütter, H. G. (1985) *Biomed. Biochim. Acta 44,* 959
Heinrich, R., Holzhütter, H. G. & Schuster, S. (1987) *Bull. Math. Biol. 49,* 539
Heinrich, R. & Rapoport, T. A. (1974*a*) *Eur. J. Biochem. 42,* 89-95
Heinrich, R. & Rapoport, T. A. (1974*b*) *Symp. Biol. Hung. 18,* 173
Heinrich, R. & Rapoport, T. A. (1975) *BioSystems 7,* 130-136
Heinrich, R., Rapoport, S. M. & Rapoport, T. A. (1977) *Progr. Biophys. Molec. Biol. 32,* 1-82
Heinrich, R. & Sonntag, I. (1982) *BioSystems 15,* 301
Higgins, J. J. (1965) in *Control of Energy Metabolism* (Chance, B, Estabrook, R. W. & Williamson, J. R., eds), pp. 13-46, Academic Press, New York, London
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65
Rapoport, T. A., Heinrich, R., Jacobasch, G. & Rapoport, S. (1974) *Eur. J. Biochem. 42,* 107
Rapoport, T. A., Heinrich, R. & Rapoport, S. M. (1976) *Biochem. J. 154,* 449
Reder C. (1986) Université de Bordeaux I, Mathématiques Appliquées, n° 8606
Reder, C. (1988) *J. theor. Biol. 135,* 175
Reich, J. G. (1984) *Biomed. Biochim. Acta 43,* 47
Reich, J. G. (1985) *Biomed. Biochim. Acta 44,* 845
Schauer, M. & Heinrich, R. (1983) *Math. Biosci. 65,* 155
Schuster, S. & Heinrich, R. (1987) *J. theor. Biol. 129,* 189
Schuster, R., Holzhütter, H. G. & Jacobasch, G. (1988) *BioSystems 22,* 19
Schuster, S. & Schuster, R. (1989) *J. Math. Chem.*, in press.
Selkov, E. E. (1975) *Eur. J. Biochem. 59,* 151
Tikhonov, A. N. (1948) *Math. Sb. 22,* 193
Waley, S. G. (1964) *Biochem. J. 91,* 514
Werner, A. & Heinrich, R. (1985) *Biomed. Biochim. Acta 44,* 185
Westerhoff, H. V. & Chen, Y. D. (1984) *Eur. J. Biochem. 142,* 425

Chapter 29

Constraints in the Application of Control Analysis to the Study of Metabolism in Hepatocytes

MICHAEL N. BERRY, ROLAND B. GREGORY, ANTHONY R. GRIVELL, DEBRA C. HENLY, JOHN W. PHILLIPS, PATRICIA G. WALLACE and G. RICKEY WELCH

IN RECENT YEARS it has been argued that quantitative methods are essential for providing new insights into the nature of the living state (Kacser, 1983). A number of mathematical approaches have evolved to meet these demands for quantitative methodologies (Heinrich & Rapoport, 1974; Savageau, 1976; Kacser & Burns, 1979), and of these, control analysis has perhaps attracted the greatest attention. Although its advent has been greeted enthusiastically by many theorists, who see it as an ideal way to quantify the regulatory role of the enzymes of a metabolic pathway, but this analytical method has not yet been widely embraced by experimentalists, because of the difficulties encountered in applying it to complex cellular systems. The work that we shall report in this chapter will illustrate some of these difficulties. The cell system we have used for our studies is the isolated hepatocyte preparation, and we shall present some representative examples from the many hundreds of experiments we have performed in this area.

Measurement of Flux Control Coefficients in Intact Hepatocytes

One approach to measuring flux control coefficients is to vary the amount of the various enzymes within a metabolic pathway. While there is a possibility of altering enzyme levels in bacteria or yeast by use of mutant species, it is extremely difficult to manipulate mammalian cells in this way. Accordingly, first Tager and colleagues (Groen *et al.*, 1982; Duszynski *et al.*, 1982) and then others (e. g. Pryor *et al.*, 1987) have adopted a more feasible procedure in which they make use of graded concentrations of specific inhibitors to

Michael N. Berry, Roland. B. Gregory, Anthony R. Grivell, Debra C. Henly, John W. Phillips and Patricia G. Wallace ● Department of Medical Biochemistry, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042 Australia ◆ G. Rickey Welch ● Department of Biological Sciences, University of New Orleans, Lake Front, New Orleans, Louisiana 70148, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

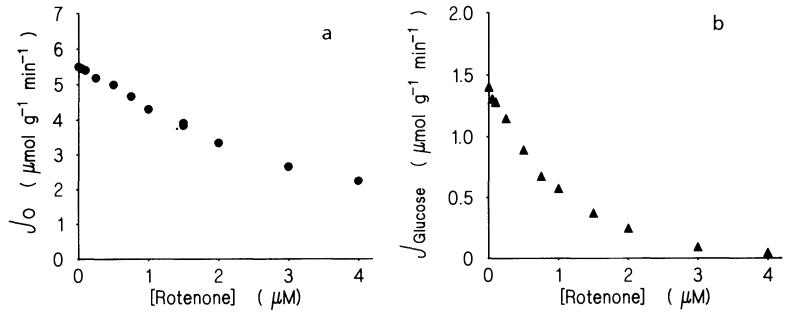


Figure 1. The effect of rotenone on (a) respiration and (b) gluconeogenesis. Hepatocytes from fasted rats were incubated with 10 mM lactate, 1 mM pyruvate and 2 mM palmitate for 35 min at 37°C as described previously (Berry *et al.*, 1983). Rates of total oxygen consumption J_O and gluconeogenesis $J_{glucose}$ are expressed in μmol min^{-1} g^{-1}.

reduce the activity of the enzyme under study. This technique has been successfully used to demonstrate the importance of the adenine nucleotide translocase in the control of mitochondrial respiration, both for the isolated organelle (Groen *et al.*, 1982) and for the intact hepatocyte (Duszynski *et al.*, 1982). Although few suitable agents are available, there are a number of inhibitors of oxidative phosphorylation or gluconeogenesis that lend themselves to whole cell work.

A good example is the irreversible inhibitor of NADH dehydrogenase, rotenone. Titration curves for this inhibitor, obtained by techniques published in a number of papers (Berry & Friend, 1969; Berry *et al.*, 1983, 1987, 1988*ab*), are provided in Fig. 1, where the influence of rotenone on respiration and gluconeogenesis is set out for hepatocytes obtained from fasted rats and incubated in the presence of lactate and palmitate. Rotenone at a concentration less than 5 μM inhibits gluconeogenesis totally and concomitantly abolishes all the extra oxygen uptake associated with lactate addition. Calculation of the respiratory control coefficient according to Duszynski*et al.* (1982) yields a value of 1. This is the inevitable outcome of performing the calculation in circumstances where the degree of inhibition of flux, at low doses of inhibitor, is a linear function of the inhibitor concentration, and where the maximal inhibitor concentration is determined by an extrapolation technique.

Not all our titrations give such a result, but it is a frequent observation particularly when examining the effects of inhibitors on the gluconeogenic pathway. Thus by means of specific inhibitors we have found flux control coefficients with respect to glucose synthesis of 1, not just for NADH dehydrogenase, but also for enolase [fluoride (Bücher, 1955)], phosphoenolpyruvate carboxykinase [mercaptopicolinate (DiTullio *etal.*,1974)] and the aspartate shuttle [aminooxyacetate (Meijer *et al.*, 1978)]. Since the summation theorem (Kacser & Burns, 1979) predicts that the sum of the flux control coefficients for the enzymes of a metabolic pathway must equal 1, these results seem paradoxical. One possibility is that we are not applying the method of control analysis correctly. A more positive interpretation is that the enzymes of the pathways under study must be so organized as to behave like a multi-enzyme complex, or to put it another way, flux must be highly "channelled".

In Fig. 2 we show a titration using the irreversible inhibitor of ATP synthase, oligo-

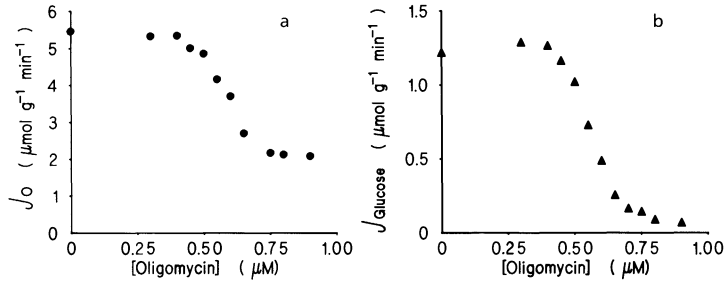


Figure 2. The effect of oligomycin on (a) respiration (J_O) and (b) gluconeogenesis ($J_{glucose}$). Hepatocytes were incubated as described in Fig 1.

mycin. It is noteworthy that significant inhibition of oxygen uptake or gluconeogenesis does not occur until the oligomycin concentration reaches 0.4 μM. This could reasonably be interpreted as indicating that an excess of ATP synthase is present under these circumstances; in other words the flux control coefficient for ATP synthesis is 0. This conclusion could be challenged, however, on the basis that in the intact cell other binding proteins might exist that would prevent oligomycin from reaching its target till a sufficiently high level of the inhibitor has been added. We can discount this possibility by noting that if ATP-dependent oxygen uptake is stimulated still further by addition of ammonia and ornithine to induce urea synthesis, the hepatocyte is capable of a much greater rate of ATP synthesis (Table 1). This would appear to confirm that the synthase is not limiting when the demand for ATP comes only from gluconeogenesis.

We have extended this study by examining the consequences of a double inhibitor titration (Kell, 1986) in which we have added to the hepatocytes amounts of oligomycin sufficient to bring about significant inhibition of respiration, and observed the additional effects of rotenone in this system. The results are unexpected (Fig. 3) in that low concentrations of rotenone invariably reduce further the oligomycin-inhibited rate of respiration and gluconeogenesis. Yet it is apparent from the rotenone titration curve in the absence of oligomycin that in the presence of these low concentrations of rotenone the hepatocytes retain sufficient respiratory capacity, that an additive effect of oligomycin and rotenone would not be anticipated. Indeed, these findings strongly support the argument for channelling of oxidative phosphorylation advanced by Kell (1986). In the context of control

Table 1. ATP requirements in hepatocytes metabolizing various substrates.

Cells were incubated for 40 min at 37°C (Berry *et al.*, 1983) at the following initial concentrations: pyruvate, 1 mM; lactate, 10mM; palmitate, 2 mM; ornithine, 2 mM; ammonium chloride 12mM. It is assumed that 6 mol ATP are required to synthesize 1 mol glucose from lactate, and 3 mol ATP to synthesize 1 mol urea from ammonia.

Substrate	J_{O_2}	$J_{glucose}$	J_{urea}	ATP required
	μmol (g wet wt.)$^{-1}$ min^{-1}			μmol/μmol
Lactate, pyruvate	3.7	0.6	–	3.6
Palmitate, lactate, pyruvate	5.5	1.2	–	7.2
Palmitate, lactate, pyruvate, ornithine, ammonia	6.8	0.9	2.3	12.1

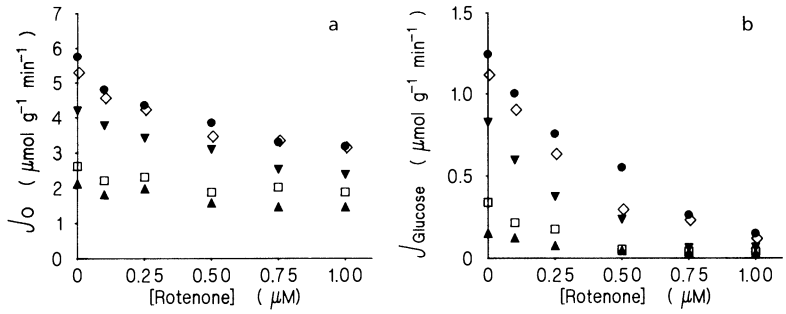


Figure 3.The effect of rotenone and oligomycin on (a) respiration (J_O) and (b) gluconeogenesis ($J_{glucose}$). Hepatocytes from fasted rats were incubated with 10 mM lactate, 1 mM pyruvate and 2 mM palmitate in the presence of various rotenone concentrations as indicated on the axis and oligomycin concentrations as follows: 0 μM (●), 0.3 μM (◇), 0.4 μM (▼), 0.5 μM (□), 0.6 μM (▲).

analysis, it will be noted that according to the linear nature of the rotenone-inhibition curves, NADH dehydrogenase continues to show a flux control coefficient of 1 for respiration and gluconeogenesis, regardless of the degree of inhibition of flux brought about by the action of oligomycin on ATP synthase.

It might reasonably be expected that since NADH dehydrogenase activity further limits respiration that has already been depressed by oligomycin, the activity of this enzyme would necessarily constrain any stimulation of respiration brought about by ATP-demand, uncoupling agents or ionophores. Yet from Table 1 it is apparent that substantial stimulation of hepatocyte respiration can be achieved when the demand for ATP is increased. Moreover, under appropriate conditions (e.g. by exposing cells to valinomycin in the presence of adequate substrate) rates of oxygen uptake as high as 14 μmol g^{-1} min^{-1} can be achieved. How then can NADH dehydrogenase exert significant control at much lower respiratory rates? We are obliged to conclude that either the analytical approach is incorrect, or alternatively that factors other than the actual amount of enzyme present are having a major influence on flux control. What these factors are does not seem to be readily revealed by control analysis.

We have also run into difficulties in endeavouring to apply the principles of control analysis to assessment of the regulatory role of the adenine nucleotide translocase. By means of control analysis (Duszynski *et al.*, 1982; Groen *et al.*, 1982) it was determined that the translocase exerts a considerable degree of control on respiratory and gluconeogenic flux in the presence of lactate. In contrast we have found a flux control coefficient of 0 at the considerably greater rates of respiration and gluconeogenesis that result when hepatocytes are incubated with lactate and palmitate in combination (results not shown, but similar to those in Fig. 2). It is by no means clear how to reconcile these discrepant findings, each based on the same technique.

It may be that the approach adopted by Groen *et al.* (1982) is not always suitable for cells as complex as hepatocytes and that other techniques should be attempted. One method is to derive flux control coefficients from elasticity coefficients rather than to measure them directly. Again, the question must be raised as to whether this can be achieved in our experimental system. There does not appear to be any certainty that the quantitative effects

of a substrate or an effector on an isolated enzyme *in vitro* will be reproduced *in vivo*, since it is not feasible to mimic exactly intracellular conditions in an *in vitro* system. Moreover, the measured concentration of a metabolite *in vivo* may be very different from its activity, due to compartmentation or to binding to intracellular components. Control analysis is theoretically able to deal with circumstances where multiple effectors are present, provided that for any given set of measurements all variables but one are held constant during the analysis. Twenty years of experience with the isolated hepatocyte preparation suggests that its inherent biological variability under physiological experimental conditions will not provide the degree of constancy required to meet such demands.

Control analysis and related approaches seem much better suited to deal with homogeneous solutions. Yet in recent years a wide body of opinion has developed that cellular organization plays a paramount role in metabolic regulation, and that enzyme-enzyme and enzyme-membrane interactions are of key importance in controlling flux (Masters, 1981; Kurganov, 1985; Welch & Clegg, 1986). As a consequence of these interactions, a change in the properties of one enzyme of a pathway can be expected to bring about significant changes in the properties of other associated enzymes. Can control analysis take into account such interactions which are so extremely difficult to emulate and analyse *in vitro*?

Glucose-Lactate Interactions

Another type of experiment that we interpret in terms of thermodynamic rather than kinetic control is illustrated in Fig. 4. Hepatocytes from fasted rats are incubated in various concentrations of glucose from 0 to 80 mM in the absence of inhibitors. No lactate is formed when glucose is not added, and in fact very little is produced when 10 mM glucose is present. With amounts of added glucose between 20 and 80 mM the initial rate of lactate production rises as a function of glucose concentration, reaching over 2 μmol g^{-1} min^{-1} at the highest glucose concentration. We conclude from discussions with María Luz Cárdenas, and our own subsequent measurements of hexokinase D ("glucokinase") activity in hepatocytes from fasted rats that the initial rates of lactate formation over the glucose range employed

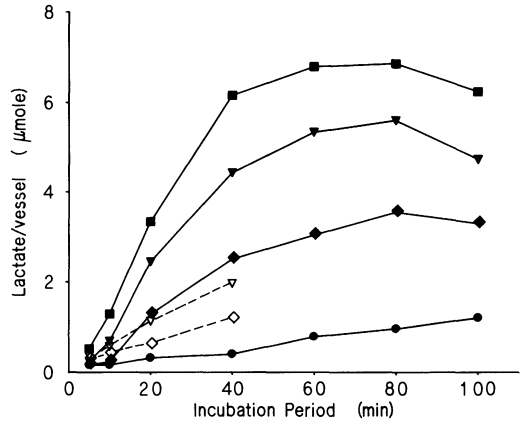


Figure 4. The effects of increasing glucose concentration on lactate formation. Hepatocytes from fasted rats were incubated with 10 mM (●), 20 mM (◆), 40 mM (▼) and 80 mM (■) glucose. NaF at 3 mM was also present in incubations containing 20 mM (◇) and 40 mM (▽) glucose.

reflect the high K_m (about 10mM in our unpublished studies) of this enzyme (see also Bontemps *et al.*, 1978).

This overall control of glycolytic flux by glucose concentration was unexpected and warranted further study. Accordingly, we examined the effects of addition to the system of 3mM sodium fluoride. This glycolytic inhibitor reduced lactate flux by two-thirds, yet, as Fig. 4 shows, the glucose concentration still played a key role in determining the rate of glycolysis. Measurement of glycolytic intermediates revealed a three-fold elevation of fructose 1,6-bisphosphate and 3-phosphoglycerate in the presence of fluoride, but no other differences were detected. It is difficult to envisage how the flux through an inhibited enzyme can be enhanced by an increase in the activity of another enzyme catalysing a reaction in the pathway some six steps removed from the site of inhibition, particularly when concentrations of metabolic intermediates along the pathway remain unchanged. The pattern seems to us more easily explained by channelling than by the diffusion and random collision of metabolites required by current formulations of control analysis.

There is another element to these studies on hepatocyte glucose metabolism, which provides even more of an interpretative challenge. It can be seen from Fig. 4 that the rates of lactate formation are not linear, but gradually decline over a period of time, reaching a steady-state after about one hour. It must be emphasized that isotope studies show conclusively that the decline in lactate accumulation is not due to the removal of lactate through the mitochondrial oxidation of pyruvate, but rather to a reduction of glycolytic flux. It is noteworthy that the final steady-state concentration of lactate in the medium, like the initial rate of lactate accumulation, is a function of the initial glucose concentration. Indeed, for concentrations of added glucose between 20 and 80 mM, the steady-state (maximum) concentration for lactate can be expressed according to the following equation:

$$[\text{glucose}] = K[\text{lactate}]^2$$

where K is found to range from 2 to 4 mM^{-1} in different experiments.

Since the hepatocyte is capable of synthesizing glucose from lactate as well as generating lactate from glucose, it was important to determine whether or not these relationships reflected a balance between glycolysis and gluconeogenesis. This was achieved by inhibiting the hepatocytes with potent inhibitors of gluconeogenesis, such as mercaptopicolinate. Addition of mercaptopicolinate did not enhance the rate of lactate accumulation nor elevate the final steady-state level reached (data not shown). Hence it can be inferred that the steady state achieved is a consequence of the glycolytic system operating alone. Nevertheless, the gluconeogenic process can play a part as is demonstrated in Fig. 5. In this experiment various amounts of lactate were added to the medium in conjunction with glucose, but the ultimate steady-state relationships between glucose and lactate were hardly affected. Where lactate in excess of the normal steady-state level was added it was removed, presumably by gluconeogenesis. When lactate, but no glucose, was added to the incubation mixture, virtually all the lactate was converted to glucose and no steady-state was achieved during the incubation period.

We have attempted to gain some insight into the processes involved by measuring the levels of metabolic intermediates. Although some of these prove technically rather difficult

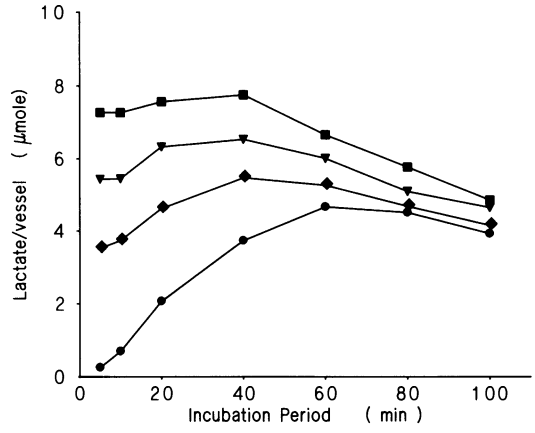


Figure 5. The effects of added lactate on lactate formation from glucose. Hepatocytes from fasted rats were incubated with 40 mM glucose (●) in the presence of 2 mM (◆), 3 mM (▼) and 4 mM (■) lactate.

to measure under our experimental conditions we can say with some degree of confidence that the achievement of a steady "plateau" in regard to lactate concentration is not accompanied by any significant change in intermediary metabolite concentrations, notably citrate, fructose 1,6-bisphosphate or adenine nucleotides (data not shown). It is difficult, therefore, to attribute changes in the glycolytic flux between 20 and 60 min to changes in the elasticity coefficients of key enzymes such as phosphofructokinase. Of course there may be subtle changes in metabolite concentrations that we have failed to detect. Nevertheless, the process appears to behave much more like a system approaching equilibrium than one under kinetic control.

It is evident, however, that the system cannot be approaching true thermodynamic equilibrium, since from the ΔG^0 of glycolysis [-112 kJ mol^{-1} (Krebs & Kornberg, 1957)], one would expect equilibrium at a [lactate]/[glucose] ratio of over 10^6. We therefore interpret our results as an example of a balance of far from equilibrium forces, where the chemical ΔG for glycolysis is balanced by an opposing intracellular force, as yet undefined. An example of such a force would be the process of electroconformational coupling described by Tsong *et al.* (1989). Such interactions could not take place in a homogeneous bulk aqueous phase, and hence we take our findings as providing compelling evidence for the existence of a high degree of cellular organization. This organization must extend to the individual enzymes (Berry *et al.*, 1987; Berry *et al.*, 1988*ab*). That is to say, intracellular metabolic flux is highly, and possibly completely, channelled.

Westerhoff & Kell (1988) were the first to discuss the breakdown of the conventional control analysis for channelling systems. The most immediate result is the violation of the unit-value flux summation. Welch *et al.* (1988) extended control analysis to heterogeneous states of enzyme organization, specifying the formal interdependence of flux-control coefficients in organized systems. Recently, Kacser *et al.* (1990; also Chapter 20 by Kacser, Sauro and Acerenza in this book) used a similar theoretical approach, in applying the analysis to homologous and heterologous enzyme-enzyme interactions. Notably, it is shown that the flux summation is *greater than unity* for channelling systems (see also Welch & Keleti, 1989). Indeed, the control-theoretical hallmark of a perfectly channelled system is a summation precisely equal to the number of enzyme steps in the pathway.

In summary, the results we have obtained in attempting to apply control analysis to our experimental data are consistent with our belief that a high degree of channelling is present. We consider that the further development of control analysis to take into account the likelihood that most metabolic pathways possess a high degree of enzyme organization, will encourage experimentalists to make more use of the method which presently appears to be exploited more frequently in the think-tank of the theorist than in the water-bath of the experimentalist.

Acknowledgements: The excellent technical assistance of Mrs. M. Grivell, Mrs. E. Williams, Miss L. Robinson, Miss V. Theunens and Miss J. Burton is gratefully acknowledged. This work was supported in part by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

Berry, M. N. & Friend, D.S. (1969) *J. Cell Biol. 43,* 506-520

Berry, M. N., Clark, D. G., Grivell, A. R. & Wallace, P. G. (1983) *Eur. J. Biochem. 131,* 205-214

Berry, M. N., Gregory, R. B., Grivell, A. R., Henly, D. C., Phillips, J. W., Wallace, P. G. & Welch, G. R. (1987) *FEBS Lett. 224,* 201-207

Berry, M. N., Gregory, R. B., Grivell, A. R., Henly, D. C., Nobes, C. D., Phillips, J. W. & Wallace, P. G. (1988*a*) *Biochim. Biophys. Acta 936,* 294-306

Berry, M. N., Gregory, R. B., Grivell, A. R., Henly, D. C., Phillips, J. W., Wallace, P. G. & Welch, G. R. (1988b) *FEBS Lett. 231*, 19-24

Bücher, T. (1955) *Methods Enzymol. 1,* 427-435

Bontemps, F., Hue, L. & Hers, H.-G. (1978) *Biochem. J. 174,* 603-611

DiTullio, N. W., Berkoff, C. E., Blank, B., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) *BiochemJ. 138,* 387-394

Duszynski, J., Groen, A. K., Wanders, R. J. A., Vervoorn, R. C. & Tager, J. M. (1982) *FEBS Lett. 146,* 116-119

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. & Tager, J. M. (1982) *J. Biol. Chem. 257,* 2754-2757

Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 97-105

Kacser, H., Sauro, H. M. & Acerenza, L. (1990) *Eur. J. Biochem.* in press

Kacser, H. & Burns, J. A. (1979) *Biochem. Soc. Trans. 7,* 1149-1160

Kacser, H. (1983) *Biochem. Soc. Trans. 11,* 35-40

Kell, D. B. (1986) *Meth. Enzymol. 127,* 538-557

Krebs, H. A., & Kornberg, H. L. (1957) *Ergebn. Physiol. 49,* 212-298

Kurganov, B. I., (1985) in *Organized Multienzyme Systems: Catalytic Properties* (Welch, G. R., ed.), pp. 241-270, Academic Press, New York

Masters, C. J. (1981) *CRC Crit. Rev. Biochem. 11,* 105-143

Meijer, A. F., Gimpel, J. A., Deleeuw, G., Tischler, M. E., Tager, J. M. & Williamson, J. R. (1978) *J. Biol. Chem. 253,* 2308

Pryor, H. J., Smyth, J. E., Quinlan, P. T. & Halestrap, A. P. (1987) *Biochem. J. 247,* 449-457

Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts

Tsong, T. Y., Liu, D-S., Chauvin, F. & Astumian, R. D. (1989) *Biosci. Rep. 9,* 13-26

Welch, G. R. & Clegg, J. S. (eds.) (1986) *The Organization of Cell Metabolism,* Plenum Press, New York

Welch, G. R., Keleti, T. & Vértessy, B. (1988) *J. Theor. Biol. 130,* 407-422

Welch, G. R. & Keleti, T. (1989) in *Structural and Organizational Aspects of Metabolic Regulation* (Srere, P., Jones, M. E. & Mathews, C., eds.) Alan R.Liss, New York

Westerhoff, H. V. & Kell, D. B. (1988) *Comments Mol. Cell. Biophys. 5,* 57-107

Chapter 30

Application of Metabolic Control Analysis to Photosynthesis: the Problem of Getting Data for an Impressive Algorithm

CHRISTOPH GIERSCH, DIRK LÄMMEL and KLAUS STEFFEN

THE MATHEMATICS of metabolic control theory is simple, not complicated as could be inferred from the literature. The differential equations describing the time course of metabolite concentrations, and qualitative knowledge of reaction rates allow formulation of two matrices. Control coefficients can then be determined from enzyme elasticities by standard matrix algebra. This procedure is used to calculate symbolically control coefficients of photosynthetic reactions. Also peculiarities of photosynthesis (light-driven reactions, high enzyme concentrations in combination with constraints) that require extension to control theory can be treated by this approach.

To obtain numerical values for control coefficients from the symbolic equations data on enzyme elasticities are required. We shall show in this chapter that the amount of data necessary for the control approach is difficult or even impossible to collect from one sample, so that original measurements have to be supplemented by data from other sources, which can lead to errors in calculated control coefficients of unknown magnitude. The integrative potential of control analysis seems to be outweighed in part by its requirement of data on the complete system. Some progress may come from expressing the ratio of control coefficients (rather than the coefficients themselves) by elasticities, which requires considerably less data. At present, control analysis is a set of impressive relations for quantities that are difficult or impossible to measure.

On the Mathematics of Metabolic Control Analysis

Calculation of control coefficients from known enzyme elasticities is a prerequisite for applying control theory to metabolic systems. An algorithm for calculation of control

Christoph Giersch and Dirk Lämmel ● Universität Düsseldorf, Institut für Biochemie der Pflanzen, Universitätsstraße 1/26.03, D-4000 Düsseldorf, Federal Republic of Germany ◆ Klaus Steffen ● Mathematisches Institut der Universität Düsseldorf, Universitätsstraße 1/25.13, D-4000 Düsseldorf, Federal Republic of Germany

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

coefficients has been proposed recently (Giersch, 1988). The essence of this algorithm is the notion that steady state metabolite concentrations are implicitly defined as functions of enzyme concentrations by the steady state condition of the system. This fact allows calculation of partial derivatives of steady state metabolite concentration with respect to enzyme concentrations, from which concentration control coefficients are obtained. This approach is sketched here for a biochemical system with m metabolites x_j and n reaction rates v_i. All reaction rates v_i are assumed to be proportional to concentration E_i of the enzyme catalysing v_i. This latter assumption is known as the central dogma of metabolic control analysis. The differential equations describing the time course of metabolite concentrations x_j are as follows:

$$\frac{dx_j}{dt} = \alpha_{j1}v_1 + \cdots + \alpha_{jn}v_n, \quad j = 1 \cdots m \tag{1}$$

α_{ji} are coefficients that indicate whether v_i is a source ($\alpha_{ji} > 0$) or a sink for x_j ($\alpha_{ji} < 0$), or is not connected to x_j ($\alpha_{ji} = 0$). Interpreting the terms on the right-hand-side of eqn. (1) not as summands but as matrix entries an $m \times n$ matrix is obtained, the weighted stoicheiometric matrix **A**:

$$\mathbf{A} = \begin{bmatrix} \alpha_{11}v_1 & \cdots & \alpha_{1n}v_n \\ \vdots & \ddots & \vdots \\ \alpha_{m1}v_1 & \cdots & \alpha_{mn}v_n \end{bmatrix} \tag{2}$$

For most biochemical systems **A** will not have maximal rank, corresponding to the fact that some of the metabolite concentrations are related by constraints involving other metabolites. How to deal with constraints has been discussed in the literature (Giersch, 1988; Reder, 1988). We note here only that each constraint on metabolite concentrations leads to deletion of a row in **A** [and to additional entries in the elasticity matrix **B**: see, e.g., the first column of **B** in eqn. (9), below]. We assume that matrix **A** has maximal rank [i.e. rank(**A**) = m], thus that the system of equations $\mathbf{A}(1 \cdots 1)^T = [\mathbf{0}]$, where $(1 \cdots 1)^T$ is the transpose of row $(1 \cdots 1)$, has $n - m$ linear independent solution vectors $(v_1 \ldots v_n)$; v_i in eqn. (2) is the ith component of the general solution to $\mathbf{A}(1 \cdots 1)^T = [\mathbf{0}]$. Note that $n - m$ is the number of independent fluxes running through the system.

A second matrix, the elasticity matrix **B**, can be formulated from knowledge of the functional dependence $v_i(x_1 \ldots x_m)$ ("which reaction rate depends on which metabolites"):

$$\mathbf{B} = \begin{bmatrix} \varepsilon_1^1 & \cdots & \varepsilon_1^n \\ \vdots & \ddots & \vdots \\ \varepsilon_m^1 & \cdots & \varepsilon_m^n \end{bmatrix} \tag{3}$$

where $\varepsilon_j^i = (x_j/v_i)(\partial v_i/\partial x_j)$ are enzyme elasticities. If no constraints are imposed on the system, and if all v_i are affected by their direct substrates and products only, **A** and **B** have entries at the same positions, i.e. matrices **A** and **B** have identical structures.

Table 1. Theorems of metabolic control analysis[1]

Theorem, written in components	as matrix	Number of equations[2]	Basis for theorem	References and notes[3]
$\sum_i C_i^{S_j} = 0$		m	Conservation of mass ($\sum \alpha_{ji} v_i = 0$)	*a,b*
$\sum_i C_i^{J_p} = 1$		n	Conservation of mass ($\sum \alpha_{ji} v_i = 0$)	*a,b*
$\sum_i C_i^{S_j} \varepsilon_k^i = -\delta_{jk}$	$[C_i^{S_j}]\mathbf{B}^T = -[1_m]$	m^2	$(\mathbf{AB}^T)^{-1}\mathbf{AB}^T = [1_m]$ and eqn. (4)	*c, d*
$\sum_i C_i^{J_p} \varepsilon_k^i = 0$	$[C_i^{J_p}]\mathbf{B}^T = [\mathbf{0}]$	mn	$(\mathbf{AB}^T)^{-1}\mathbf{AB}^T = [1_m]$ and eqn. (5)	*a*
$\sum_i \alpha_{ji} v_i C_p^{J_r} = 0$	$\mathbf{A}[C_i^{J_p}] = [\mathbf{0}]$	mn	$\mathbf{AB}^T(\mathbf{AB}^T)^{-1} = [1_m]$ and eqn. (5)	*e*
$\sum_i C_i^{J_r} = n - m$	$\mathrm{tr}[C_i^{J_p}] = n - m$	1	$\mathrm{tr}\{(\mathbf{AB}^T)^{-1}\mathbf{AB}^T\} = \mathrm{tr}\{\mathbf{B}^T(\mathbf{AB}^T)^{-1}\mathbf{A}\} = m$	*f,g*
$\sum_i C_i^{S_j} C_p^{J_r} = 0$	$[C_i^{S_j}][C_i^{J_p}] = [\mathbf{0}]$	-	Right-hand sides of eqns. (4-5)	*h*

[1]The metabolic pathway is assumed to have n enzymes and m free metabolites whose steady state concentrations are S_j. The number of independent fluxes running through the system is $n - m$ and equals the trace of the $n \times n$ matrix $[C_p^{J_r}]$ of flux control coefficients.

[2]The number of equations that allow calculation of control coefficients from enzyme elasticities.

[3]*a*, Kacser & Burns (1973); *b*, Heinrich & Rapoport, 1974; *c*, Westerhoff & Chen, 1984; *d*, δ_{jk} is the Kronecker δ, equal to 1 if $j = k$, to 0 if $j \neq k$; *e*, Reder, 1988; *f*, Giersch, 1988; *g*, tr(**M**) is the trace of matrix **M**; *h*, published here for the first time.

The essential assumptions for application of the implicit function theorem are that system (1) has a steady state and that the $m \times m$ matrix $\mathbf{AB}^T$ evaluated at the steady state is invertible ($\mathbf{B}^T$ is the transpose of **B**). Necessary conditions for invertibility of $\mathbf{AB}^T$ are discussed in Giersch (1988). If the inverse $(\mathbf{AB}^T)^{-1}$ exists, the mn concentration control coefficients $C_i^{S_j}$ and the n^2 flux control coefficients $C_i^{J_p}$ can be calculated from the following pair of equations:

$$[C_i^{S_j}] = -(\mathbf{AB}^T)^{-1}\mathbf{A} \tag{4}$$

$$[C_i^{J_p}] = [1_n] - \mathbf{B}^T(\mathbf{AB}^T)^{-1}\mathbf{A} \tag{5}$$

in which $[1_n]$ in eqn. (5) is the $n \times n$ unit matrix. The theorems of metabolic control analysis (Table 1) follow from the steady-state condition ($\sum_i \alpha_{ji} v_i = 0$, so row sums of $(\mathbf{AB}^T)^{-1}\mathbf{A}$ and $\mathbf{B}^T(\mathbf{AB}^T)^{-1}\mathbf{A}$ are zero), and from the particular structures in terms of **A** and **B** of the right-hand-sides of eqns. (4) and (5), as outlined in Table 1. An additional theorem, $[C_i^{S_j}][C_i^{J_p}] = [\mathbf{0}]$, follows also immediately from eqns. (4-5). Note that the seven theorems hold for any pair (**C, D**) of matrices as long as $(\mathbf{CD}^T)$ is invertible and the row sums of **C** are zero.

Control coefficients calculated from eqns. (4-5) are uniquely determined (because of the implicit function theorem). What about uniqueness of coefficients obtained from the theorems of Table 1? There are $m(m + 1)$ equations for calculation of the mn concentration control coefficients $C_i^{S_j}$, so that calculation does not give unique results if $n - m > 1$.

The n^2 flux control coefficients $C_i^{J_p}$ are to be calculated from $1 + n(2m + 1)$ equations (Table 1), so that unique results are not obtained if $n - 2m > 1$. The difference $n - m$, the number of independent fluxes, is determined by the topological structure of the system. Calculation of control coefficients for systems with high $n - m$ by means of the theorems of Table 1 requires additional branch point relationships (see Fell & Sauro, 1985, Westerhoff & Kell, 1987). These are not needed for the calculation by eqns. (4-5). On the other hand, if uniqueness is guaranteed, calculation of flux control coefficients by means of the theorems is more convenient than by eqn. (5). In any case, calculation of algebraic expressions for control coefficients in terms of enzyme elasticities requires relatively simple matrix algebra only. For small systems, symbolic calculation of control coefficients can be done with paper and pencil.

Although the theorems of control analysis follow immediately from eqns. (4-5), some points of interest remain: are there more theorems than the seven listed in Table 1? Note that e.g. $\sum_j \alpha_{ji} v_i = 0$ (column sums of **A** are zero) except for columns corresponding to reactions at the "ends" of the pathway. Could this lead to additional theorems? Moreover, the theorems of Table 1 hold for any elasticity matrix **B** (as long as $\mathbf{A}\mathbf{B}^T$ is invertible) whereas pathway structures and rate laws cause **B** (and **A**) to adopt certain characteristic properties. What are the consequences in terms of control coefficients of these characteristic structures of matrices **A** and **B**?

Before applying the above algorithm to photosynthesis, two peculiarities of photosynthetic CO_2 fixation are considered: light driven reactions and enzymes occurring at high (mM) concentrations.

Control Analytical Treatment of Peculiarities of Photosynthesis

Light-Driven Reactions. Peculiar to photosynthesis are light-driven reactions like electron transport or photophosphorylation. The rate R of a light reaction is dependent both on light intensity L and on the biochemical capacity R_m of the plant: $R = R(L, R_m)$. For light-driven reactions, the two parameters L and R_m substitute for the single parameter (enzyme concentration) in ordinary biochemical reactions. An extension of control theory to allow analysis of light reactions is to assume that R is proportional to (or homogeneous of degree 1 in) L and R_m: $R(tL, tR_m) = tR(L, R_m)$ for any t, which, from Euler's theorem on homogeneous functions, is equivalent to

$$\frac{L}{R}\frac{\partial R}{\partial L} + \frac{R_m}{R}\frac{\partial R}{\partial R_m} = 1 \tag{6}$$

We make the following definitions:

$$h_L = \frac{L}{R}\frac{\partial R}{\partial L};\quad h_{R_m} = \frac{R_m}{R}\frac{\partial R}{\partial R_m} \tag{6a}$$

so that $h_L + h_{R_m} = 1$ from Euler's relation, eqn. (6). h_L and h_{R_m} are the sensitivities of rate R towards changes in light L and biochemical capacity R_m. It can be shown (Giersch *et al.*, 1990) that control coefficients for light C_L and the biochemical capacity C_{R_m} can be obtained by multiplying by h_L and h_{R_m}, respectively, the coefficient C calculated as for

ordinary biochemical reactions. In our analysis of photosynthetic CO_2 fixation we use a model of a light-driven reaction that has been proposed by Farquhar & Wong (1984). It has the advantage of being homogeneous of degree 1 in L and R_m and is given by:

$$0.67R^2 - R(R_m + q \cdot L/2) + R_m \cdot q \cdot L/2 \quad (7)$$

where R is the rate of the light-driven reaction and q the fraction of light absorbed by chloroplasts.

High Enzyme Concentration. At least one of the enzymes of photosynthetic CO_2 fixation occurs at concentration comparable to that of its substrate: the concentration of active sites of ribulose-1,5-bisphosphate carboxylase in the chloroplast stroma has been estimated to be about 4 mM, whereas the concentration of its substrate ribulose 1,5-bisphosphate can be considerably lower (the other substrate of ribulose-1,5-bisphosphate carboxylase, CO_2, is treated as a parameter). While in an open system the occurrence of enzymes at high concentrations does not violate the central dogma of metabolic control analysis (see Chapter 20 by Kacser, Sauro and Acerenza in this book) this is not true when additional constraints are imposed on one of the metabolites turned over by the highly concentrated enzyme. As ribulose 1,5-bisphosphate participates in the phosphate pool in the chloroplast stroma, which is known to be constant, the reaction rate v of ribulose-1,5-bisphosphate carboxylase is not a homogeneous function of the enzyme concentration E. From Euler's theorem (or from inspection of the rate law of this enzyme: see Farquhar, 1979) non-homogeneity of v in E is equivalent to $E\partial v/v\partial E \neq 1$. Non-homogeneity has the consequence that the theorems of control analysis (Table 1) do not hold. This affects also interpretation of control coefficients (what does a flux control coefficient of 0.8 mean if the sum of flux control coefficients is certainly not equal to 1 but otherwise unknown?). However, control coefficients can be redefined in such a way that all theorems of Table 1 hold for the redefined set even if $E\partial v/v\partial E \neq 1$ (Giersch *et al.*, 1990). The "new" coefficients, K_i, are simply obtained by dividing the "old" ones by $E\partial v/v\partial E$:

$$K_i^J = C_i^J \Big/ \frac{E_i}{v_i}\frac{\partial v_i}{\partial E_i}, \quad K_i^{S_j} = C_i^{S_j} \Big/ \frac{E_i}{v_i}\frac{\partial v_i}{\partial E_i}$$

so that $K_i = C_i$ if (and only if) $E\partial v/v\partial E = 1$. The K_i coefficients are a generalization of the C_i coefficients and can be calculated in the same way as the latter from eqns. (4-5) or by means of the theorems.

Elasticity Matrix and Stoicheiometric Matrix for the Calvin Cycle

The pathway of photosynthetic CO_2 fixation involves about 25 reactions and metabolites. A simplified model of the pathway with only six reactions is shown in Fig. 1. This model highlights some of the basic principles of CO_2 fixation: CO_2 is bound to the acceptor ribulose 1,5-bisphosphate, which is regenerated in a circular ATP-requiring process. Triose phosphate from the chloroplast is exchanged for inorganic phosphate in the cytosol. The model

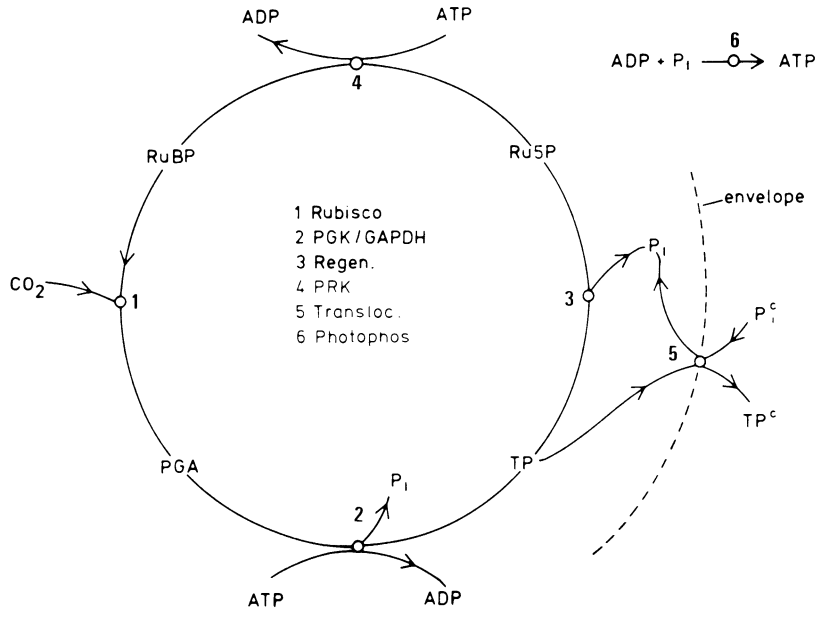


Figure 1. Simplified model of the Calvin cycle. This model features photophosphorylation (v_6) as a light-driven reaction, the phosphate translocator (v_5), and four reactions (v_1 - v_4) modelling CO_2 fixation and regeneration of ribulose 1,5-bisphosphate: v_1, ribulose-1,5-bisphosphate carboxylase; v_2, phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase; v_3, regeneration of pentose phosphates; v_4, phosphoribulokinase. The rate law for ribulose-1,5-bisphosphate carboxylase (Farquhar, 1978) considers that the concentration of this enzyme is of the same order as ribulose 1,5-bisphosphate. The model has seven variables (ribulose 1,5-bisphosphate ... ATP) which are interrelated by two constraints (conservation of adenylates, conservation of total phosphate in the chloroplast) such that there are 5 free variables; the concentrations of CO_2, inorganic phosphate (P_i) and triose phosphate (TP) in the cytosol are parameters. The superscript c denotes species in the cytosol. Other abbreviations: TP, triose phosphate; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglycerate.

has seven metabolites (CO_2, as well as triose phosphate and inorganic phosphate in the cytosol, are parameters). The differential equations for the system of Fig. 1 are as follows:

$$
\begin{aligned}
\frac{d}{dt}[\text{ATP}] &= 0 - v_2 + 0 - v_4 + 0 + v_6 \\
\frac{d}{dt}[\text{3-}P\text{-glycerate}] &= 2v_1 - v_2 + 0 + 0 + 0 + 0 \\
\frac{d}{dt}[\text{triose-}P] &= 0 + v_2 - 5v_3 + 0 - v_5 + 0 \\
\frac{d}{dt}[\text{ribulose 5-}P] &= 0 + 0 + 3v_3 - v_4 + 0 + 0 \\
\frac{d}{dt}[\text{inorganic }P] &= 0 + v_2 + 2v_3 + 0 + v_5 - v_6 \\
\frac{d}{dt}[\text{ADP}] &= 0 + v_2 + 0 + v_4 + 0 - v_6 \\
\frac{d}{dt}[\text{ribulose-1,5-}P_2]_t &= -v_1 + 0 + 0 + v_4 + 0 + 0
\end{aligned}
\qquad (8)
$$

$[\text{ribulose 1,5-}P_2]_f$ is the concentration of free ribulose 1,5-bisphosphate. The total concentration of ribulose 1,5-bisphosphate exceeds the free concentration by the concentration of the

(ribulose-1,5-bisphosphate carboxylase)•(ribulose 1,5-bisphosphate) complex. The right-hand-sides of the rows in eqn. (8) are interpreted as rows of a matrix **A'** (see above). Inspection of **A'** shows that its rank is not maximal: the second to last row is opposite in sign to the first one ([ATP] + [ADP] = const.), and the last one can be expressed in terms of rows 1 to 5 (the total phosphate concentration, organic + inorganic, in the chloroplast stroma is constant). Matrix **A**, the weighted stoicheiometric matrix, is obtained by deleting from **A'** the last two rows. **A** (but not **A'**) has maximal rank; thus the number of independent fluxes is 6 - 5 = 1. The components (v_1 ... v_6) of the flux vector are $v_1(1, 2, 0.33, 1, 0.33, 3)$.

Formulation of the elasticity matrix **B** requires knowledge of the rate laws v_i. It is assumed here that all reaction rates v_i are affected only by their direct substrates or products. Reactions 1, 3, 4, and 6 are formulated as irreversible reactions because of their high Gibbs energy change. Thus, the functional dependence $v_i = v_i(x_j)$ is described as follows (see Giersch *et al.*, 1990, for details): $v_1 = v_1([\text{ribulose 1,5-}P_2]_t)$; $v_2 = v_2([\text{3-phosphoglycerate}], [\text{ATP}], [\text{ADP}], [\text{triose phosphate}], [\text{inorganic phosphate}])$; $v_3 = v_3([\text{triose phosphate}])$; $v_4 = v_4([\text{ribulose 5-phosphate}], [\text{ATP}])$; $v_5 = v_5([\text{inorganic phosphate}], [\text{triose phosphate}])$; $v_6 = v_6([\text{ADP}], [\text{inorganic phosphate}])$. Denoting metabolites by their row numbers in (8) the elasticity matrix B can be written as follows:

$$\mathbf{B} = \begin{bmatrix} \varepsilon_1^1 & \varepsilon_1^2 & 0 & \varepsilon_1^4 & 0 & \varepsilon_1^6 \\ \varepsilon_2^1 & \varepsilon_2^2 & 0 & 0 & 0 & 0 \\ \varepsilon_3^1 & \varepsilon_3^2 & \varepsilon_3^3 & 0 & \varepsilon_3^5 & 0 \\ \varepsilon_4^1 & 0 & 0 & \varepsilon_4^4 & 0 & 0 \\ \varepsilon_5^1 & \varepsilon_5^2 & 0 & 0 & \varepsilon_5^5 & \varepsilon_5^6 \end{bmatrix} \tag{9}$$

(note that $v_1 = v_1(x_1 \cdots x_5)$ due to the constraint that P_0, the total phosphate concentration in the stroma, expressed as $x_1 + \cdots + x_5 + [\text{ribulose 1,5-}P_2]_t$, is conserved). From eqns. (8-9) the 5 x 5 matrix $\mathbf{AB}^T$ is calculated. Only four of its 25 entries are zero. It is assumed that $\mathbf{AB}^T$ evaluated at steady state values of v_i and ε_k^i is invertible. Though somewhat laborious, the inverse, $(\mathbf{AB}^T)^{-1}$, can be calculated using paper and pencil (or, with less effort, by a computer program capable of performing symbolic calculations). Control coefficients K_i are then expressed in terms of α_{ji}, ε_k^i, and v_i by means of eqns. (4) and (5). Corresponding matrices A and B for the complete photosynthetic pathway can be formulated in analogous manner. They are 21 x 23 matrices (25 metabolites with 4 constraints, 23 enzymes).

Above we have shown that symbolic determination of control coefficients from enzyme elasticities is relatively simple even for complicated pathways like that of photosynthetic CO_2 fixation. Another issue, considered now, is the search for data on enzyme elasticities.

Estimation of Control Coefficients for Photosynthetic Reactions

To calculate numerical values for the K_i, data on enzyme elasticities are required. Two approaches are considered: (*a*) the more conventional one of constructing a simulation model, and (*b*) the direct one of collecting data on $\partial v_i/\partial x_j$.

(a) Using data on reaction kinetics $v_i(x_j, p_k)$ and solving a nonlinear system of equations. There is a large volume of published data on the kinetics of Calvin cycle enzymes. For the majority of enzymes, there exist at least estimates of Michaelis and inhibition constants and limiting rates. These data allow formulation of reaction rates v_i (for details see Giersch *et al.*, 1990). However, published data on photosynthetic reactions are not complete and often inconsistent. We have estimated missing data by means of the simulation model of eqn. (8). Steady-state metabolite concentrations were calculated by solving system (8) of non-linear equations for S_j, from which steady-state fluxes and enzyme elasticities are obtained. Control coefficients are then calculated according to eqns. (4-5).

Fig. 2 shows the dependence of calculated metabolite concentrations and flux control coefficients on the activity of phosphoribulokinase. Decreasing the activity of this enzyme below about 2.5 mM/s (or 230 μmol/[mg chlorophyll•h]) leads to classical manifestation of flux limitation at the phosphoribulokinase reaction: the products ribulose 1,5-bisphosphate, 3-phosphoglycerate and triose phosphate decline while substrate ribulose 5-phosphate is piling up. Flux control coefficients K_i behave as expected: that for reactions 1 to 3 decrease, and that for the phosphoribulokinase reaction (K_4) increases with decreasing phospho-

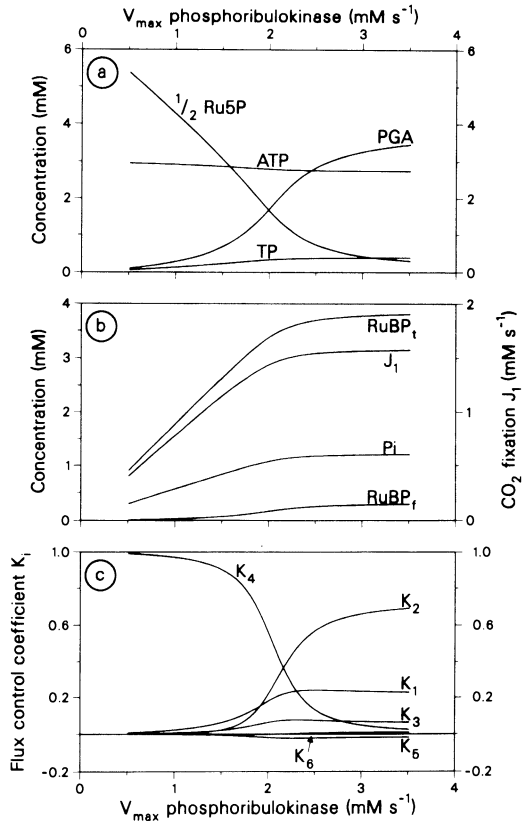


Figure 2. Dependence of calculated steady state metabolite concentrations (*a*, *b*) and flux control coefficients (*c*) on phosphoribulokinase activity. J_1 is the rate of CO_2 fixation (1mM s^{-1} is equal to 90 μmol per mg chlorophyll per hour). Parameter values for this simulation correspond to saturating light and nearly saturating CO_2. The components of K_6 [see eqn. (6a) above] are not denoted as they almost coincide, but see Fig. 3. $RuBP_{t(f)}$: total (free) ribulose 1,5-bisphosphate concentration; other abbreviations as in Fig. 1.

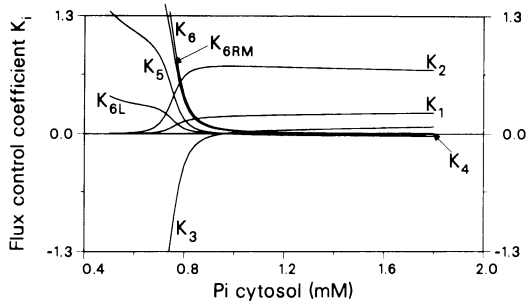


Figure 3. Dependence of calculated flux control coefficients K_i on the concentration of inorganic phosphate in the cytosol. Note the "sensitivity catastrophe" when this concentration is less than 0.8 mM, where the absolute values of K_3, K_5 and K_6 exceed 1.3. The summands of K_6, $K_{6L}=h_L \cdot K_6$ and $K_{6RM}=h_{RM} \cdot K_6$ [see eqn. (6a)] are also shown, from which it is evident that photophosphorylation is more sensitive to an increase in the biochemical capacity (R_m) than to an increase in light. Parameter values are as for Fig. 2 except V_{max} of phosphoribulokinase, which is 4.81 mM s^{-1}.

ribulokinase activity (Fig. 2). Note that $|K_i| \leq 1$ for all K_i. The limiting rate of phosphoribulokinase observed under physiological conditions is high (corresponding to at least 5 mM/s) such that the flux control coefficient of phosphoribulokinase can be estimated from Fig. 2 to be insignificant.

Dependence of flux control coefficients on external phosphate (cytosol inorganic phosphate) is depicted in Fig. 3. It is known that photosynthesis is a process requiring inorganic phosphate, which is taken up from the cytoplasm in exchange for triose phosphate from the chloroplast. Therefore, it is expected that the calculated concentration of inorganic phosphate in the chloroplast stroma, and as a consequence (Giersch & Robinson, 1987), the ATP concentration and the rate of CO_2 fixation, decline when the concentration of inorganic phosphate in the cytoplasm decreases (not shown). Flux control coefficients show dramatic changes when this concentration falls below 0.9 mM: that for the phosphate translocator (K_5) and photophosphorylation (K_6) increase drastically, while that for regeneration of pentose phosphates from triose phosphates (K_3) declines to large negative values. Note that the absolute values of K_3, K_5 and K_6 exceed 1 at low concentrations of inorganic phosphate in the cytoplasm (Fig. 3).

Increase in K_5 with decreasing concentrations of inorganic phosphate in the cytoplasm reflects the limiting capacity of the phosphate translocator to facilitate diffusion of inorganic phosphate and phosphate esters ("no import of inorganic phosphate into the chloroplast if the concentration of inorganic phosphate in the cytoplasm is low"), and increase in K_6 reflects the inorganic phosphate requirement of photophosphorylation ("no phosphorylation without inorganic phosphate"). Why is the control coefficient for K_3 negative and its modulus so high (Fig. 3)? An increase in activity of reaction 3 (Scheme 1) will further decrease triose phosphate and inorganic phosphate in the chloroplast (the concentration control coefficient of triose phosphate and inorganic phosphate towards v_3 are -5.7 and -4.5, respectively) and thereby lower the rate of inorganic phosphate import and of ATP synthesis. Thus, at low external phosphate, the triose phosphate consuming regeneration of pentose phosphates exerts a kind of "supercontrol" on the flux. It would be interesting to check if this result of the model can be verified experimentally.

The examples of Figs. 2 and 3 show that complete "sensitivity scenarios" can be studied if rate laws are used for formulation of elasticity matrix **B**. While this approach certainly

aids understanding of qualitative aspects of regulation, the present incompleteness of data on reaction rates v_i causes the numbers calculated for control coefficients to be subject to discussion. They should be considered with caution. Whereas we calculated the flux control coefficient of ribulose-1,5-bisphosphate carboxylase at 1000 μmol quanta m^{-2} s^{-1} and nearly saturating CO_2 to be close to 0.2 (Fig. 3), Woodrow (1986) concluded that "... ribulose-1,5-bisphosphate carboxylase may, under certain conditions, be a major controller" of photosynthesis which probably means $0.3 < K_1 < 0.8$, and Pettersson & Ryde-Pettersson (1988) presented a model indicating that K_1 is close to zero at saturating CO_2. Available data do not allow yet to give preference to any of these figures.

(*b*) *Using data on the derivatives* $\partial v_i/\partial x_j$. This approach starts from experimental data on S_j, J_i and the partial derivatives $\partial v_i/\partial x_j$, measured at the steady state. Metabolite concentrations and fluxes can be measured by conventional methods. One of the problems with this approach comes from the fact that all data have to be sampled at one specific steady state. For the simple model of Fig. 1, twelve partial derivatives have to be determined (besides the flux and the metabolite concentrations), for the complete scheme (21 metabolites, 23 enzymes) more than 50 partial derivatives. Given the daily variation of plant material, sufficient data for the steady state in question are difficult or even impossible to collect. However, the required amount of data can be reduced drastically if the analysis is restricted to ratios of control coefficients rather then to the coefficients themselves. For example, assuming the functional dependence $v_i(x_j)$ as for the model of Fig. 1, the ratio K_1/K_6 of flux control coefficients for ribulose-1,5-bisphosphate carboxylase and photophosphorylation can be shown (Giersch *et al.*, 1990) to be as follows:

$$\frac{K_1}{K_6} = \frac{0.66 \cdot \dfrac{\partial v_6}{\partial [\text{ADP}]}}{\dfrac{\partial v_1}{\partial [\text{ribulose-1,5-}P_2]_t}\left(\dfrac{\dfrac{\partial v_2}{\partial [\text{ADP}]}}{\dfrac{\partial v_2}{\partial [\text{3-}P\text{-glycerate}]}} - 1\right)} \qquad (10)$$

so that measurement of four partial derivatives is sufficient to determine the ratio K_1/K_6. From published data the partial derivatives in eqn. (10) can be estimated to be about 1.52, 0.26 (3 mM ribulose 1,5-bisphosphate) or 0.0064 (5 mM ribulose 1,5-bisphosphate), 0.157 and 1.29 (all s^{-1}) for $\partial v_6/\partial$[ADP], $\partial v_1/\partial$[ribulose-1,5-P_2]$_t$, $\partial v_2/\partial$[ATP], and $\partial v_2/\partial$[3-phosphoglycerate], respectively (saturating illumination and ambient CO_2). The ratios K_1/K_6 calculated from these data are 0.54 and 22 for total ribulose 1,5-bisphosphate concentrations of 3 mM and 5 mM, respectively (note that K_1/K_6 is about 20 for nearly saturating CO_2 according to Fig. 2). What is the "correct" ribulose 1,5-bisphosphate concentration? Its total concentration, like that of other metabolites, can vary at least twofold in parallel experiments. The drastic dependence of calculated K_1/K_6 ratios on assumed values of this concentration implies that all data have to be sampled from the same batch of plant material. This again demonstrates that calculation of control coefficients from pooled data extracted from the literature cannot produce reliable results.

The major problem with this approach is measurement of $\partial v_i/\partial x_j$. *In vitro* data that are known to reflect the *in vivo* situation only partially can be collected from the isolated enzyme by established methods. However, under certain assumptions regarding the functional dependence $v_i(x_j)$, even *in vivo* data on the partial derivatives can be obtained: $\partial v_i/\partial x_j$ can be expressed by $\partial J_i/\partial p$ and $\partial S_j/\partial p$, where p is an external parameter like the CO_2 concentration. The two latter derivatives can be obtained from *in vivo* measurements of J_i and S_j for various values of p, so that it seems feasible to determine *in vivo* elasticities without isolating enzymes or measuring enzyme activities. No such data exist yet, and it is obvious that the price one has to pay in terms of experimental effort is high.

Conclusion

Control analysis has developed impressive relations between parameter sensitivities of steady state concentrations or of fluxes on the one hand and enzyme elasticities on the other. These relations allow systemic properties to be expressed in analytical form by local ones. For small systems and for systems with simple structures (linear metabolic chains, ecological food chains) clues on modulus and sign of control coefficients can be derived solely from qualitative knowledge of the rate laws. This is valuable information.

Calculation of numbers for control coefficients of a pathway like the Calvin cycle requires collection of experimental data on all fluxes, all metabolite concentrations, and reaction kinetics of all enzymes of the pathway for one given steady state of the plant material. For reasons outlined above this type of experimental data is difficult or impossible to obtain. Collecting data for calculating ratios of control coefficients seems more feasible, since fewer data are required. Nevertheless, it cannot be overlooked that calculation of control coefficients of photosynthetic reactions from enzyme elasticities is at present hampered by the lack of suitable methods for collecting experimental data of the type required for control analysis.

REFERENCES

Farquhar, G. D.(1979) *Arch. Biochem. Biophys. 193,* 456-468
Farquhar, G. D. & Wong, S. C. (1984) *Aust. J. Plant Physiol. 11,* 191-210
Fell, D. A. & Sauro, H. M. (1985) *Eur. J. Biochem. 148,* 555-561
Giersch, C. & Robinson, S. P. (1987) *Photosynth. Res. 14,* 211-227
Giersch, C. (1988) *J. Theor. Biol. 134,* 451-462
Giersch, C., Lämmel, D. & Farquhar, G. D. (1990), *Photosynth. Res.* in press
Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 89-95
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Pettersson, G. & Ryde-Pettersson, U.(1988) *Eur. J. Biochem. 175,* 661-672
Reder, C. (1988) *J. Theor. Biol. 135,* 175-201
Westerhoff, H. V. & Chen, Y.(1984) *Eur. J. Biochem. 142,* 425-430
Westerhoff, H. V. & Kell, D. B. (1987) *Biotech. Bioeng. 30,* 101-107
Woodrow, I. E. (1986) *Biochim. Biophys. Acta 851,* 181-192

Chapter 31

Application of Control Analysis to Photosynthetic Sucrose Synthesis

MARK STITT

SUCROSE is the major product of photosynthesis, and it is exported to the rest of the plant via the phloem. However, a substantial portion of the photosynthate is retained in the leaf, often as starch. This starch is later degraded to provide respiratory substrate, or to support continued synthesis of sucrose for export during the night. Since major increases in crop productivity have been related to an improved allocation of photosynthate within the plant rather than an increase in the basic rate of photosynthesis (Gifford *et al.*, 1984), there is considerable interest in understanding the mechanisms which regulate the partitioning of photosynthate, and in identifying sites which might allow this partitioning to be manipulated.

In this chapter we shall describe some experiments using mutants of *Clarkia xantiana* with a step-wise reduction in the activity of either the cytosolic or the chloroplast phosphoglucose isomerase. These mutants have allowed us to selectively change the rate of sucrose or starch synthesis, and study the importance of the mechanisms which are thought to be involved in regulating these fluxes. By applying the concepts of Kacser & Burns (1973), we have made a first attempt at describing the control of partitioning quantitatively, in terms of the flux control coefficients and elasticity coefficients of some of the enzymes involved.

Background — Pathways and Regulatory Mechanisms for Sucrose and Starch Synthesis

Current research suggests that the partitioning of photosynthate is controlled from the cytosol (Stitt *et al.*, 1987*b*). CO_2 is initially fixed via the Calvin cycle in the chloroplast, and triose-phosphates are then exported to the cytosol (Fig. 1). The inorganic phosphate released during sucrose synthesis then returns to the chloroplast, where it is needed to support further photophosphorylation. The export of triose phosphates actually occurs in a strict counter-exchange with inorganic phosphate, which is catalysed by the phosphate translocator (Flügge & Heldt, 1984). When sucrose synthesis is restricted, less inorganic

Mark Stitt ● Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth, 8580 Bayreuth, Federal Republic of Germany

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

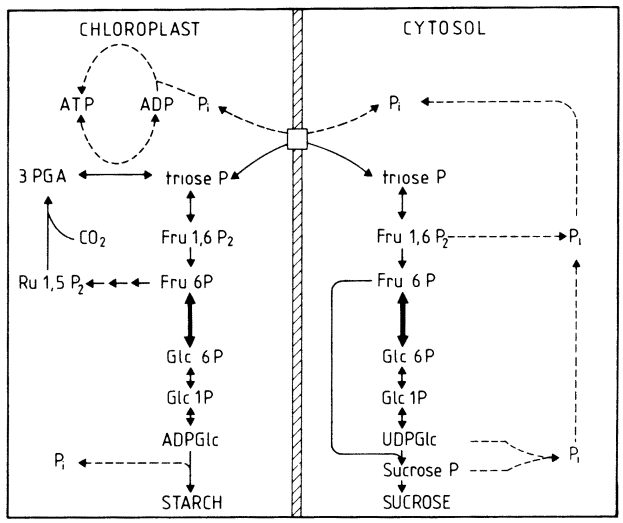


Figure 1. Pathways of Photosynthetic Carbon Metabolism. Fluxes of carbon are shown as solid lines, and fluxes of inorganic phosphate as dotted lines. For simplicity UTP and pyrophosphate are omitted from the reaction catalysed by UDPglucose pyrophosphorylase, and UDP is omitted from the reaction catalysed by sucrose phosphate synthase. Individual reactions involved in the regeneration of ribulose 1,5-bisphosphate have also been omitted.

phosphate is released in the cytosol, and the export of triose-phosphate is decreased. More photosynthate is then retained in the chloroplast and converted to starch, recycling inorganic phosphate within the chloroplast to support further photosynthesis

Techniques are available to measure the subcellular metabolite levels in protoplasts (Wirtz *et al.*, 1980; Lilley *et al.*, 1982; Gardeström & Wigge, 1988) or by non-aqueous density gradient centrifugation of lyophilized leaf material (Gerhardt *et al.*, 1987). It has been shown that four reactions in the cytosol are substantially removed from their thermodynamic equilibrium, viz. the cytosolic fructose 1,6-bisphosphatase, sucrose phosphate synthase, sucrose-6-phosphatase and the hydrolysis of pyrophosphate (Gerhardt *et al.*, 1987; Weiner *et al.*, 1987; Quick *et al.*, 1989). The remaining reactions are all very close to equilibrium. Research during the last 6 years has also established that the cytosolic fructose 1,6-bisphosphatase and sucrose phosphate synthase are regulated to allow the rate of photosynthesis to be (*a*) increased in response to a rising rate of photosynthesis and (*b*) decreased in response to a lower demand for sucrose, allowing more photosynthate to be temporarily stored as starch in the chloroplast (Stitt *et al.*, 1987*ab;* Stitt & Quick, 1989).

Two factors are thought to be important for the short-term regulation of sucrose phosphate synthase. Firstly, sucrose phosphate synthase has an allosteric site, with glucose 6-phosphate acting as an activator and inorganic phosphate as an inhibitor. This means that a rising supply of hexose-phosphate will act to stimulate sucrose synthesis (Doehlert & Huber, 1984). Secondly, evidence is accumulating that sucrose phosphate synthase is regulated via covalent protein modification. This involves changes of the kinetic properties (Stitt *et al.*, 1988; Walker & Huber, 1988), and preliminary evidence suggests it may involve protein

phosphorylation (Huber *et al.*, 1989). This levels to an activation of sucrose phosphate synthase as the rate of photosynthesis increases (Stitt *et al.*, 1988).

The cytosolic fructose 1,6-bisphosphatase is regulated (inhibited) by the regulator metabolite fructose 2,6-bisphosphate (Herzog *et al.*, 1984; Stitt *et al.*, 1987*a*). The fructose 2,6-bisphosphate concentration depends upon the activity of the enzymes which are responsible for its synthesis and degradation, fructose 6-phosphate 2-kinase and fructose 2,6-bisphosphatase, which themselves are regulated by metabolites. Rising rates of photosynthesis lead to an increase of 3-phosphoglycerate and triose-phosphates and, probably, a decrease of inorganic phosphate. These act to inhibit fructose 6-phosphate 2-kinase, and the resulting decrease of fructose 2,6-bisphosphate then deinhibits the cytosolic fructose 1,6-bisphosphatase (Stitt *et al.*, 1987*a*). The rising triose-phosphate concentration also leads to large increase of the substrate, fructose 1,6-bisphosphate, because these metabolites are linked by a second-order reaction, and to a yet larger activation of the cytosolic fructose 1,6-bisphosphatase, because this enzyme has a sigmoidal substrate saturation curve (Herzog *et al.*, 1984; Stitt *et al.*, 1987*a*).

A model has been developed to describe the response of the cytosolic fructose 1,6-bisphosphatase to a rising supply to triose-phosphate as the rate of photosynthesis increases (Herzog *et al.*, 1984; Stitt & Heldt, 1985*b*; Stitt *et al.*, 1987*a*). The model takes into account the measured reciprocal changes of the inhibitor (fructose 2,6-bisphosphate) and the substrate, as well as the properties of the partially purified enzyme. It predicts that the fructose 1,6-bisphosphatase will be effectively inactive until a "threshold" concentration of triose-phosphate is exceeded, above which the activity rises very sharply (Fig. 2). This prediction is in good agreement with the measured relation between the triose-phosphate level and the rate of sucrose-synthesis in leaves (see Fig. 2).

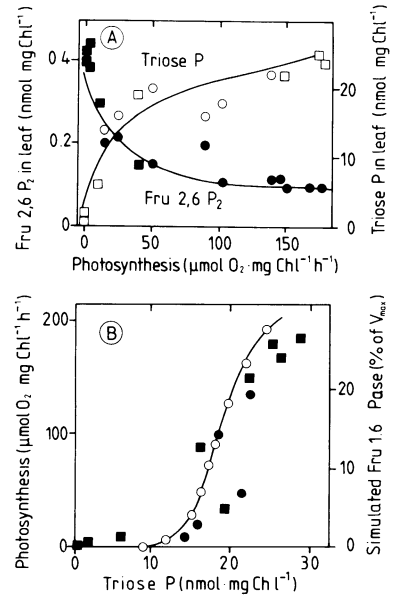


Figure 2. A model for the regulation of the cytosolic Fru1,6Pase in response to a rising supply of triose-phosphate. A, Changes of fructose 2,6-bisphosphate (Fru 2,6 P_2) and triose phosphate (Triose P) as the rate of photosynthesis increases. B, Comparison of the modelled response of the cytosolic fructose 1,6-bisphosphatase (○ and line) and the measured relation between the triose-phosphate content and the rate of photosynthesis as the light intensity (■) or the CO_2 concentration is increased (●). The cytosolic fructose 1,6-bisphosphatase activity was modelled from the kinetic properties of the partially purified enzyme and the measured levels of fructose 2,6-bisphosphate and triose-phosphates in leaves, assuming the reactions catalysed by triose-phosphate isomerase and aldolase were close to equilibrium (see Stitt & Heldt, 1985*b*; Stitt *et al.*, 1987*a*).

This highly cooperative response of the fructose 1,6-bisphosphatase *in vivo* is very important, because rapid CO_2 fixation requires a rather delicate balance between the turnover of the Calvin cycle and the removal of triose-phosphate. If too much triose-phosphate were to be removed for end product synthesis, photosynthesis would be inhibited because the regeneration of ribulose 1,5-bisphosphate (the acceptor for CO_2) would be prevented. On the other hand, if triose-phosphates were to be removed too slowly, photophosphorylation and photosynthesis would be inhibited because too little inorganic phosphate would be recycled. Inactivation of the fructose 1,6-bisphosphatase below a "threshold" level of triose-phosphates, presumably reflecting the level needed for Calvin cycle turnover, will ensure a first priority for the regeneration of more acceptor. The sharp activation of the fructose 1,6-bisphosphatase after this "threshold" is exceeded will ensure that the "surplus" triose phosphate is rapidly removed and converted to sucrose, recycling inorganic phosphate to support photosynthesis (Stitt *et al.*, 1987*ab*). Incidentally, the absolute value of the "threshold" can be altered to allow rapid intercellular diffusion of metabolites during photosynthesis in specialized C-4 plants like maize (Stitt & Heldt, 1985*a*), and as an adaptation to decreasing temperature (Stitt & Große, 1988).

So far we have considered some of the mechanisms which allow the rate of sucrose synthesis to be coordinated with the rate of CO_2 fixation. Balancing regulatory mechanisms also operate in the cytosol to allow a feedback inhibition of sucrose synthesis and alter partitioning towards starch. When sucrose accumulates in the leaf or is supplied exogenously, sucrose phosphate synthase is inactivated (Stitt *et al.*, 1988) and hexose-phosphates increase in the cytosol (Gerhardt *et al.*, 1987). The increased fructose 6-phosphate then activates fructose 6-phosphate 2-kinase and inhibits fructose 2,6-bisphosphatase (Stitt *et al.*, 1984*a;* Larondelle *et al.*, 1986; MacDonald *et al.*, 1989), leading to an increase of fructose 2,6-bisphosphate (Stitt *et al.*, 1983; Stitt *et al.*, 1984*b*) and, thence, to an inhibition of the cytosolic fructose 1,6-bisphosphatase (Gerhardt *et al.*, 1987). More photosynthate is then retained in the chloroplast for conversion to starch.

The mechanisms that are directly responsible for stimulating starch synthesis have been studied in isolated chloroplasts, where the effect of decreased sucrose synthesis can be stimulated by decreasing the concentration of inorganic phosphate that is supplied in the medium in which the chloroplasts are suspended. Decreased inorganic phosphate leads to a lower ATP/ADP ratio, and the reduction of 3-phosphoglycerate is restricted (Heldt *et al.*, 1977). The resulting increase of the ratio of 3-phosphoglycerate to inorganic phosphate then activates ADP-glucose pyrophosphorylase (Preiss, 1985) and starch synthesis is increased.

These studies used what might be termed the "traditional" approach to the study of regulation, concentrating on (*a*) identifying the "irreversible" reactions and then (*b*) studying the changes of metabolites and fluxes *in vivo* and (*c*) the properties of the isolated enzymes in the hope that (*d*) a regulatory mechanism could be proposed. This approach, however, has now led to a dilemma. In the course of these studies it became obvious that there are several enzymes in the cytosol which could be contributing to regulation, (for example, we have not even considered sucrose-phosphatase or the removal of pyrophosphate, see Quick *et al.*, 1989; Stitt & Quick, 1989) and it also became obvious that several mechanisms could be operating to regulate each enzyme; for example, the cytosolic

fructose 1,6-bisphosphatase could also be regulated by AMP (Herzog *et al.*, 1984), and possibly by calcium (M. Brauer, M. Stitt & D. Sanders, unpublished).

We therefore began to suspect that the "traditional" approach was not going to allow us to clearly define the relative importance of these various enzymes and mechanisms for the control of flux. Firstly, the approach involves basically, the study of correlations and it is notoriously difficult to assign causality to correlations. Secondly, the pathway and the individual enzymes were becoming very complex and while a complete "reductionist" description for a given cultivar in fixed growth conditions might be possible, it was doubtful whether it would be feasible to use this approach for a wide range of plants or growth conditions. For these reasons, we have recently become interested in the possibility of using mutants with a progressive reduction in the activity of a chosen enzyme. These might allow a more rigorous analysis of these regulatory mechanisms and possibly, a first application of control theory to these pathways.

Studies using Mutants with a Reduced Activity of the Cytosolic or the Chloroplast Phosphoglucose Isomerase

The experiments were carried out with mutants of *Clarkia xantiana* which had a step-wise reduction in the activity of the cytosolic (Jones *et al.*, 1986*a*) or the chloroplastic (Jones *et al.*, 1986*b*) phosphoglucose isomerase. The role of these two enzymes during photosynthetic metabolism can be seen from Fig. 1. The chloroplast phosphoglucose isomerase is essential for starch synthesis, and the cytosolic phosphoglucose isomerase is needed for sucrose synthesis. Although neither is directly involved in the Calvin cycle, there is an indirect dependence because CO_2 fixation requires sucrose and starch synthesis to recycle inorganic phosphate. We have used these mutants to investigate the following questions:

1. Can these mutants be used to directly measure the flux control coefficient of the cytosolic and plastid phosphoglucose isomerase? On one hand, we were interested in assessing the technical difficulties in this kind of study. We were also interested in using this as a test case for the widespread assumption that enzymes like phosphoglucose isomerase, which catalyse a "near-equilibrium" reaction, are in "excess" and cannot control flux through a pathway.

2. Does a decrease of the cytosolic and chloroplast phosphoglucose isomerase have an analogous effect on the fluxes, or is there an asymmetrical interaction between the chloroplast and the cytosol? Photosynthetic carbon metabolism is, essentially, a branched pathway leading, after the Calvin cycle, to either sucrose or starch. Obviously, the phosphoglucose isomerase isoenzyme in one subcellular compartment will have a flux control coefficient for the pathway leading to the end-product in its own compartment. It would also have a flux control coefficient for the end-product synthesis in the other compartment, which will probably be "negative", because decreasing phosphoglucose isomerase in one branch will tend to divert photosynthate into the other branch of the

pathway. Each phosphoglucose isomerase will also have a flux control coefficient for photosynthesis, whose magnitude will depend upon the ability of one branch to respond to a decreased flux in the other branch. Current notions about the control of partitioning suggest that the rate of starch synthesis responds to changes in the rate of starch synthesis, rather than *vice versa*. In that case, we might expect a different pattern to emerge, depending upon whether the chloroplast or the cytosol phosphoglucose isomerase is decreased.

3. Can the cytosolic phosphoglucose isomerase mutants be used to directly test and quantify the contribution that fructose 2,6-bisphosphate makes to the control of photosynthate partitioning? Decreased cytosolic phosphoglucose isomerase should lead to an increase of fructose 6-phosphate, which (see above) is thought to be responsible for the increase of fructose 2,6-bisphosphate and, thence, the inhibition of the cytosolic fructose 1,6-bisphosphatase. The cytosolic mutants should allow us to test and quantify the importance of these interactions.

We approached these questions by illuminating leaves in saturating ^{14}C-labelled CO_2, and measuring the flux to sucrose and starch via ^{14}C-incorporation. Parallel samples were quenched in liquid N_2, extracted, and assayed for metabolites. The experiments were carried out in limiting and in saturating light, to allow different fluxes to be imposed through the pathways. The detailed results of the experiments (Kruckeberg *et al.*, 1989; Neuhaus *et al.*, 1989) and a theoretical treatment of the pathway of sucrose synthesis (Stitt, 1989*ab*) have been published elsewhere.

Is Phosphoglucose Isomerase "in Excess"? Determination of the Flux Control Coefficients, Disequilibrium Ratios and Elasticity Coefficients

Measurement of flux control coefficients depends upon being able to accurately measure small changes in the metabolic fluxes. A lower limit of resolution is set, however, by the variability of these kind of measurements, as the standard error is often about 10% of the mean. These studies also depend on the quality of the mutants. Even after taking the plant material through a rigorous crossing programme to produce an isogenic background, it is still possible that the original mutation has pleiotropic effects on the expression of other genes. Hopefully, these effects will be minimized when mutants are used which have relatively little effect on growth rates, as was the case in these studies.

The effects of decreased phosphoglucose isomerase on the fluxes to starch and sucrose and the mass action ratio are summarized in Fig. 3 and Table 1. The effect of each isoenzyme on the fluxes in its own compartment will be discussed first (i.e. the effect of decreased plastid phosphoglucose isomerase on starch synthesis, and of decreased cytosolic phosphoglucose isomerase on sucrose synthesis; for discussion of the effect on the other fluxes, see below). It is apparent that the effect on flux depends (*a*) on the subcellular isoenzyme and (*b*) on the conditions.

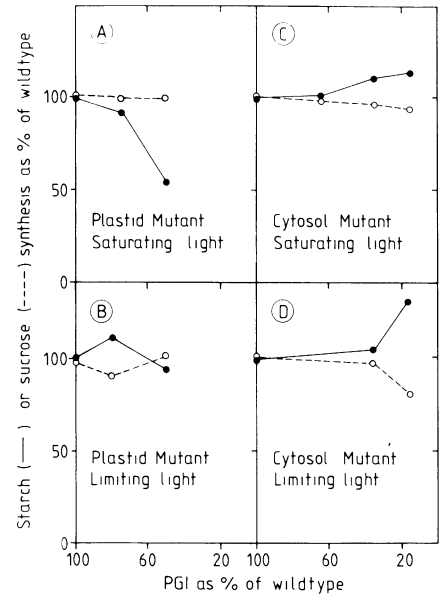


Figure 3. Enzyme-flux relationship for phosphoglucose isomerase and photosynthetic carbon metabolism. A, Plastid phosphoglucose isomerase against fluxes at saturating light. B, Plastid phosphoglucose isomerase against fluxes at limiting light. C, Cytosolic phosphoglucose isomerase against fluxes at saturating light. D, Cytosolic phosphoglucose isomerase against fluxes at limiting light. The fluxes to sucrose (○) and starch (●) are shown.

The chloroplast phosphoglucose isomerase has a significant control coefficient for starch synthesis in the wildtype in high light (about 0.34), but it has no consistent or significant effect on flux in low light (Fig. 3). The mass action ratio also decreases markedly below the expected equilibrium constant (about 3.3) in high light, but was not significantly affected in low light (Table 1). Thus reduced chloroplast phosphoglucose isomerase activity has a similar effect on the local reaction parameters and on flux through the pathway, and phosphoglucose isomerase makes an increasing contribution to control as the flux imposed on the pathway is increased.

A more complex picture emerges for the cytosolic phosphoglucose isomerase. The mass

Table 1. Influence of decreased plastid or cytosolic phosphoglucose isomerase activity on the ratio of fructose 6-phosphate to glucose 6-phosphate*

Phosphoglucose isomerase activity (as % of wild type)		[Glucose 6-*P*]/[Fructose 6-*P*]	
Plastid	Cytosol	Saturating light	Limiting light
100	100	2.61 ± 0.06	2.87 ± 0.06
75	100	2.17 ± 0.07	2.78 ± 0.08
50	100	1.86 ± 0.08	2.76 ± 0.07
100	64	2.43 ± 0.04	2.92 ± 0.04
100	36	2.23 ± 0.05	2.61 ± 0.06
100	18	1.54 ± 0.09	2.19 ± 0.09

*Leaves from four separate plants (120-200 μg chloroplasts) were illuminated at 1000 μmol m^{-2} s^{-1} (saturating) or 125 μmol m^{-2} s^{-1} (limiting) in saturating CO_2 at 15°C for 10 min before transferring them rapidly into liquid N_2 with illumination being continued at the same light intensity. The results are mean ± standard error of the mean from 4-8 separate experiments.

action ratio decreases continuously as cytosolic phosphoglucose isomerase activity is reduced, with the decrease being apparent in low light and larger in high light (Table 1). However, there is negligible effect on fluxes until two-thirds of the cytosolic phosphoglucose isomerase has been removed and, when an effect does appear, the effect is larger in low light than in high light (Fig. 3). The flux control coefficient of the cytosolic phosphoglucose isomerase therefore (*a*) responds in an opposite manner to the chloroplast phosphoglucose isomerase as the light intensity is increased, and (*b*) is not related to the extent of disequilibrium.

The measurements of fructose 6-phosphate and glucose 6-phosphate allowed the elasticity coefficients of phosphoglucose isomerase to be estimated (Table 2). Again, the variability in the data (standard error about 10% of the mean) places constraints on the accuracy of these estimates. However, for an enzyme like phosphoglucose isomerase, which may reasonably be assumed to have hyperbolic substrate saturation kinetics, the equations developed by Groen *et al.* (1982) can be used. They have the advantage that the glucose 6-phosphate/fructose 6-phosphate ratio can be used, instead of the absolute metabolite levels. This is useful, because the variability in the metabolite ratios is less than the variability in the individual metabolite levels (see Kruckeberg *et al.*, 1989).

Table 2. Estimated elasticity coefficients *in vivo* for the cytosolic phosphoglucose isomerase

Phosphoglucose isomerase activity (as % of wild type)	Fructose 6-*P*		Glucose 6-*P*	
	High light	Low light	High light	Low light
100	4.6	7.6	-4.6	-6.9
64	3.2	8.4	-3.3	-8.4
36	2.3	5.2	-2.5	-4.9
18	1.2	2.6	-1.5	-2.1

The elasticity coefficients of the cytosolic phosphoglucose isomerase for fructose 6-phosphate and glucose 6-phosphate are between 4.5 and 7.5 in the wild type (Table 2), emphasizing that "near equilibrium" reactions typically have relatively high elasticities for their substrate and product. This is not due to any inherent kinetic property of the enzyme; rather, the high elasticities arise because the net activity v represents the difference between the forward (v_{+1}) and the reverse (v_{-1}) reaction (Kacser & Burns, 1979). If v_{+1} and v_{-1} are of a similar magnitude, then a relatively small change of e.g. v_{+1} caused by a change of the substrate concentration will lead to a considerably larger change in the net flux, (v_{+1} - v_{-1}).

Table 2 also shows that the elasticity coefficients fall progressively as phosphoglucose isomerase is decreased in the mutants. When less phosphoglucose isomerase is available, the reaction will have to be further displaced from equilibrium to maintain a given net reaction rate. This means that v_{+1} and v_{-1} will diverge, and the net activity (v_{+1} - v_{-1}) will become less sensitive to e.g. a small change of v_{+1} caused by a change of the substrate concentration.

The cytosolic phosphoglucose isomerase starts to adopt a significant flux control

coefficient in low light when the elasticity coefficients for its substrate and product fall to about 2. At this point, the reaction has a disequilibrium ratio of about 0.66, or a free energy change of only -1.25 kJ mol^{-1} (see Kruckeberg *et al.*, 1989; Stitt, 1989*b*). Clearly, a reaction can exert control under some circumstances even when it is still near to equilibrium. Equally, it is clear that phosphoglucose isomerase is not present in "excess". The chloroplast phosphoglucose isomerase can have a significant flux control coefficient even in the wild type. The cytosol phosphoglucose isomerase activity is barely adequate to maintain its reactants reasonably close to equilibrium, and can also start to exert control when its activity is reduced by more than 3-4 fold.

Asymmetrical Interactions between the Branches of the Pathway

The interaction between the fluxes in the cytosol and the chloroplast is markedly asymmetrical. When the rate of starch synthesis is decreased by reduced chloroplast phosphoglucose isomerase activity, there is no significant compensatory increase of sucrose synthesis (Fig. 3). Instead, the rate of photosynthesis decreases. A similar result has recently been obtained using a pea mutant with a decreased amount of the starch branching enzyme (Smith, Neuhaus & Stitt, 1990).

In contrast, in mutants with a reduced cytosolic phosphoglucose isomerase, the decreased rate of sucrose synthesis is fully compensated for by an increased rate of starch synthesis and the rate of photosynthesis is no significantly affected. The cytosolic phosphoglucose isomerase therefore adopts a significant negative flux control coefficient for starch synthesis in these mutants, rising to about -0.21 in low light. In high light, there is less effect on the flux to sucrose and the flux to starch is also less affected.

These results are fully consistent with the notion that partitioning is regulated from the cytosol, rather than the chloroplast. They also emphasize that partitioning can be altered without this necessarily impairing the overall rate of photosynthesis. Indeed, the effect of reduced phosphoglucose isomerase activity on flux (but not on the local parameters) is largely overridden in conditions which allow high rates of photosynthesis. This behaviour has an obvious functional importance. It means that sucrose synthesis can be decreased to allow more photosynthate to be redirected towards storage as starch but can, nevertheless, be increased again if more favourable conditions permit a higher rate of photosynthesis. This will minimize the risk that an adjustment of partitioning leads to a decreased rate of photosynthesis. The question now arises, how this flexible response in the cytosol is achieved.

Quantification of the Fructose 2,6-Bisphosphate Regulator Cycle

The influence of reduced phosphoglucose isomerase activity on the activity of the cytosolic fructose 1,6-bisphosphatase will depend upon (*a*) the effect of an increase of fructose 6-phosphate on the fructose 2,6-bisphosphate concentration and (*b*) the effectiveness of fructose 2,6-bisphosphate as an inhibitor of the cytosolic fructose 1,6-bisphosphatase.

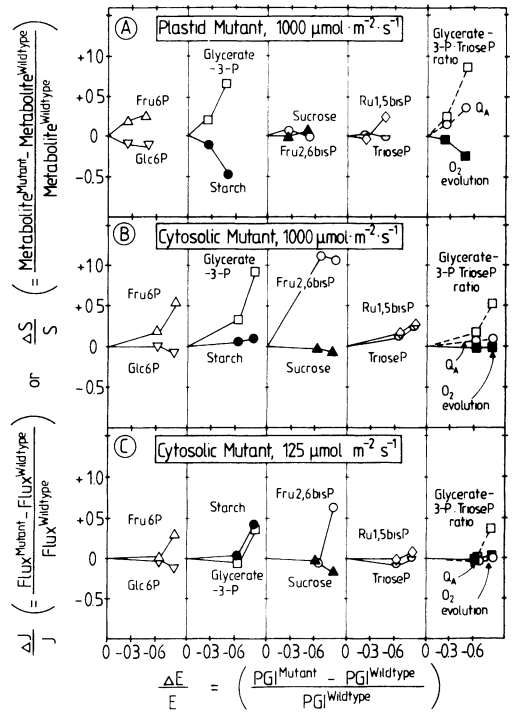


Figure 4. Enzyme-flux and enzyme-metabolite relation in *Clarkia* mutants with A, reduced chloroplast or B, C, reduced cytosolic phosphoglucose isomerase. The results for the cytosolic series are given for B, high and C, low light. Fluxes are shown as solid symbols for starch (●) and sucrose (▲) synthesis, and the overall rate of photosynthesis (■). Metabolites are shown as open symbols including fructose 6-phosphate (Δ), Glc6P (∇) 3-phosphoglycerate (❑), fructose 2,6-bisphosphate (○), ribulose 1,6-bisphosphate (◊), triose phosphate (∪), reduction of Q_A (the primary acceptor for photosystem 2, (○) and the 3-phosphoglycerate/triose-phosphate ratio (❑).

When cytosolic phosphoglucose isomerase is reduced, the increase of fructose 6-phosphate is accompanied by a 2-3 fold larger increase of fructose 2,6-bisphosphate (Fig. 4). A similar relation is seen when fructose 6-phosphate increases in leaves as sucrose accumulates (Stitt, 1989*ab*). This relation can be represented by an amplification factor α, and it has been shown (Stitt, 1989*a*) that α can also be expressed in terms of the properties of the enzymes of this regulator cycle, such that

$$\alpha = \frac{d[\text{Fru-2,6-}P_2]}{[\text{Fru-2,6-}P_2]} \Big/ \frac{d[\text{Fru-6-}P]}{[\text{Fru-6-}P]} = \frac{\varepsilon^{2\text{-K}}_{\text{Fru-6-}P} - \varepsilon^{2\text{-P}}_{\text{Fru-6-}P}}{\varepsilon^{2\text{-P}}_{\text{Fru-2,6-}P_2} - \varepsilon^{2\text{-K}}_{\text{Fru-2,6-}P_2}}$$

where $d[\text{Fru-2,6-}P_2]/[\text{Fru-2,6-}P_2]$ and $d[\text{Fru-6-}P]/[\text{Fru-6-}P]$ are the fractional changes in the steady state concentrations of fructose 2,6-bisphosphate and fructose 6-phosphate respectively, and the symbols ε represent the elasticity coefficients of fructose 6-phosphate 2-kinase (2-K) for fructose 2,6-bisphosphate and fructose 6-phosphate, and of fructose 2,6-bisphosphatase (2-P) for fructose 2,6-bisphosphate and fructose 6-phosphate. Analysis of the kinetic properties of the partially purified enzymes suggests that α could approach a maximal value of about 4, provided the fructose 2,6-bisphosphatase does not become substrate-saturated. This is a reasonable assumption, because the major fructose 2,6-bisphosphatase in the cytosol has a K_m for fructose 2,6-bisphosphate of at least 30 µM (MacDonald *et al.*, 1989), which is much higher than the fructose 2,6-bisphosphate concentration of 2-10 µM

(Stitt *et al.*, 1983; 1984*b*; 1987*a*). The empirical estimates for the gain in this regulator cycle are therefore in quite reasonable agreement with the properties of the enzymes which are involved.

The impact of the increased fructose 2,6-bisphosphate on the flux to sucrose varies, depending on the conditions (Fig. 3). In low light, a 50% increase of fructose 2,6-bisphosphate leads to a 20% decrease of sucrose synthesis and a 30% increase of starch synthesis. In high light, the fructose 2,6-bisphosphate level doubled, but starch and sucrose synthesis only changed by about 10%. In interpreting the experiments in high light, it might be noted that the cytosolic fructose 1,6-bisphosphatase has a sigmoidal substrate response (see above). The increased levels of triose-phosphate in the mutant in saturating light (Fig. 3C) could therefore explain why flux is only slightly affected by increased fructose 2,6-bisphosphate in these conditions.

This interaction can be treated more quantitatively. Fructose 1,6-bisphosphatase activity can be represented by the following simplified expression:

$$\frac{dv}{v} = \frac{d[\text{Fru-2,6-}P_2]}{[\text{Fru-2,6-}P_2]} \varepsilon^{\text{Fru-1,6-bisphosphatase}}_{\text{Fru-2,6-}P_2} + \frac{d[\text{triose-}P]}{[\text{triose-}P]} \varepsilon^{\text{Fru-1,6-bisphosphatase}}_{\text{triose-}P}$$

where the symbols ε represent the elasticity coefficients of the cytosolic fructose 1,6-bisphosphatase for fructose 2,6-bisphosphate and triose-phosphates. This expression treats triose-phosphate isomerase, aldolase and fructose 1,6-bisphosphatase as a block of reactions. It also omits the term describing the interaction with the product (fructose 6-phosphate) because the reaction is irreversible, and because there is no product inhibition in the presence of physiological concentrations of fructose 2,6-bisphosphate (see Stitt, 1989*a*).

The data in Fig. 4 can then be used to estimate elasticities by the dual modulation method (Kacser & Burns, 1979). The equations developed by Groen *et al.* (1982) are not applicable in this case, because the fructose 2,6-bisphosphatase is markedly non-hyperbolic. The dual modulation method can be applied in this case, because the elasticity coefficients are somewhat lower than for phosphoglucose isomerase, and the estimates are therefore less susceptible to experimental error in the metabolite and flux determinations. Nevertheless, the values should only be regarded as approximations. The estimates also only represent an *average* of the elasticities found over the conditions used (i.e. in this case, high and low light; wildtype and reduced phosphoglucose isomerase activity). Obviously, a small perturbation is theoretically desirable but, experimentally, fairly large perturbations have to be used to obtain a change in the metabolite level or flux which can be reliably measured.

The data in Fig. 4 yield estimates for the elasticity coefficients for triose-phosphates of +1.5 and fructose 2,6-bisphosphate of -0.4. These agree quite well with estimates of +1.1 and -0.5 which have been obtained by analysis of the perturbations of metabolites and fluxes in spinach leaves in response to small changes in the light intensity and CO_2 concentration, or the amount of sucrose in the leaf (Stitt, 1989*a*). They also agree quite well with the values which can be predicted from the *in vitro* kinetics of the partially purified cytosolic fructose 1,6-bisphosphatase when it is operating in conditions where it is partially saturated with substrate (Herzog *et al.*, 1984; Stitt, 1989*a*).

These results emphasize therefore (*a*) that an increase of fructose 6-phosphate will lead to a 2-3 fold amplified increase of fructose 2,6-bisphosphate, (*b*) that fructose 2,6-bisphosphate will inhibit the cytosolic fructose 2,6-bisphosphatase and (*c*) that the increase of fructose 2,6-bisphosphate can be overriden by a relatively much smaller increase of the triose-phosphates. Thus, in low light, an increase of fructose 2,6-bisphosphate is quite effective in changing partitioning. In saturating light when high rates of CO_2 fixation and 3-phosphoglycerate reduction are achieved, this restriction of sucrose synthesis can, if necessary, be overcome by a relatively small increment in the triose-phosphate pool.

Application of the Connectivity Theorem to Investigate the Distribution of Control

The question now arises why the flux control coefficient of phosphoglucose isomerase is smaller in high light than in low light. The impact of reduced phosphoglucose isomerase will depend upon the relation between the elasticity coefficients of phosphoglucose isomerase for fructose 6-phosphate and glucose 6-phosphate, and the elasticity coefficients that other, controlling, enzymes have for these metabolites, as stated in the connectivity theorem (Kacser & Burns, 1973). The decreased flux control coefficient of phosphoglucose isomerase in high light could therefore be explained if (*a*) there is a drastic decrease in the elasticity coefficients of other enzymes for fructose 6-phosphate or glucose 6-phosphate or (*b*) if control is distributed away from other enzymes which happen to respond most sensitively to fructose 6-phosphate or glucose 6-phosphate.

This can be illustrated by considering the cytosolic fructose 1,6-bisphosphatase. The fructose 1,6-bisphosphatase does not respond directly to fructose 6-phosphate (see above), but it is affected indirectly via the change of fructose 2,6-bisphosphate. Accordingly, a notional elasticity for fructose 6-phosphate is given by the expression $\alpha \cdot \varepsilon_{Fru\text{-}2,6\text{-}P_2}^{Fru\text{-}1,6\text{-}bisphosphatase}$, which has a value of about -0.8 (averaged over the conditions used in the experiments). For comparison, $\varepsilon_{Fru\text{-}6\text{-}P}^{glucose\text{-}P\ isomerase}$ varies between 4.6 to 1.2, and 7.6 to 2.6, as phosphoglucose isomerase activity is reduced in high and low light, respectively (Table 2). Given that phosphoglucose isomerase has a flux control coefficient for sucrose synthesis of 0.21 and about 0.05 in low and high light, respectively, and applying a modified form of the connectivity theorem,

$$\frac{C_{Fru\ 1,6\text{-}bisphosphatase}^{sucrose\ synthesis}}{C_{sucrose\text{-}P\ synthase}^{sucrose\ synthesis}} = -\frac{\varepsilon_{Fru\text{-}6\text{-}P}^{Glc\text{-}P\ isomerase}}{\alpha \cdot \varepsilon_{Fru\ 2,6\text{-}P_2}^{Fru\ 1,6\text{-}bisphosphatase}}$$

we can estimate that the fructose 1,6-bisphosphatase may have a flux control coefficient for sucrose synthesis of about 0.5 in low light, but only about 0.07 in high light.

A model of sucrose synthesis (Stitt, 1989*a*) has also suggested that control may usually be equally distributed between fructose 1,6-bisphosphatase and the block of reactions around sucrose phosphate synthase, but could be redistributed towards the latter block at high flux rates. This redistribution is partly due to the ability of a rising supply of triose-phosphate to override control at the fructose 1,6-bisphosphatase in conditions allowing high rates of CO_2

fixation. The effect of reduced phosphoglucose isomerase activity on the reactions clustered around sucrose phosphate synthase is likely to be weaker than the effect on the fructose 1,6-bisphosphatase. Although glucose 6-phosphate is an activator of sucrose phosphate synthase (see above), fructose 6-phosphate is actually one of the substrates of sucrose phosphate synthase, and the concentration of the other substrate, UDPglucose, can be controlled independently of the glucose 6-phosphate and glucose 1-phosphate concentration, by regulating turnover of pyrophosphate and the uridine nucleotides (Quick *et al.*, 1989; J. Dancer, H. E. Neuhaus & M. Stitt, unpublished work).

However, it should be stressed that the expressions which are used to estimate *in vivo* elasticities have to include simplifying assumptions, as well as the implicit assumption that the most important regulatory mechanisms have already all been discovered. The accuracy of these estimates is also constrained by the statistical quality of the data for fluxes and metabolite levels. Probably, conclusions based on interpretation of elasticities should be considered as tentative, pending a more direct determination of the flux control coefficients using mutants of the individual enzymes.

Outlook

Photosynthesis provides a very attractive system for applying the concepts of Kacser and Burns because (*a*) the fluxes involved can be measured relatively easily, (*b*) it is easy to perturb the system from outside by altering the light intensity or CO_2 concentration, or the export of sucrose from the leaf and (*c*) quite a wide range of mutants are already available from mutagenesis programmes. For example, we are now carrying out experiments with mutants four out of the five enzymes involved in the pathway of starch synthesis. This should allow a detailed study of the distribution of control in this important metabolic pathway. It is rather more difficult to obtain mutants of the enzymes involved in the Calvin cycle or sucrose synthesis because there is no simple screen available, and such mutants would probably often be lethal. However, there is good reason to hope that techniques like antisense DNA can be used to produce transgenic plants with a reduced amount of selected enzymes (see Rodermel *et al.*, 1988). This will make it possible to carry out a detailed analysis of the control of flux in these pathways, which are of primary importance for controlling the rate and partitioning of photosynthate. It is to be hoped that application of this combination of genetics and biochemistry will revolutionize the way in which plant metabolism is studied, as well as our ability to subsequently utilize this knowledge.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (SFB 137). I am grateful to U. Küchler for typing the manuscript, and to Dr. P. Quick, Dr. J. Dancer, A. L. Kruckeberg, Dr. L. D. Gottlieb and H. E. Neuhaus for many discussions, and their essential contribution to the experimental work described in this chapter.

REFERENCES

Doehlert, D. C. & Huber, S. C. (1984) *Plant Physiol. 76,* 250-253
Flügge, U.-I. & Heldt, H. W. (1984) *Trends Biochem. Sci. 9,* 530-533
Gardeström, P. & Wigge, B. (1988) *Plant Physiol. 88,* 69-76

Gerhardt, R., Stitt, M. & Heldt, H. W. (1987) *Plant Physiol. 83,* 399-407
Gifford, R. M., Thorne, J.H., Hitz, W. & Giaquinta, R.T. (1984) *Science 225,* 801-808
Groen, A. K., Van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, T. P. M. & Tager, J. M. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), Academic Press, London
Heldt, H. W., Chon, C. J., Maronde, D., Herold, A., Stankovic, Z. S., Walker, D. A., Krammer, A., Kirk, M. R. & Heber, U. (1977) *Plant Physiol. 59,* 1146-1155
Herzog, B., Stitt, M. & Heldt, H. W. (1984) *Plant Physiol. 75,* 561-565
Huber, J. L. A., Huber, S. C. & Nielsen, T. H. (1989) *Arch. Biochem. Biophys. 270,* 681-690
Jones, T. W. A., Pichersky, E. & Gottlieb, L. D. (1986*a*) *Genetics 113,* 101-114
Jones, T. W. A., Pichersky, E. & Gottlieb, L. D. (1986*b*) *Plant Physiol. 81,* 367-371
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-107
Kacser, H. & Burns, J. A. (1979) *Biochem. Soc. Trans. 7,* 1149-1161
Kacser, H. & Porteous, J. W. (1987) *Trends Biochem. Sci. 12,* 5-14
Kruckeberg, A. L., Neuhaus, H. E., Feil, R., Gottlieb, L. D. & Stitt, M. (1989) *Biochem. J. 261,* 457-467
Larondelle, Y., Mertens, E., Van Schaftingen, E. & Hers, H. G. (1985) *Eur. J. Biochem. 161,* 351-357
Lilley, T. McC., Stitt, M., Mader, G. & Heldt, H. W. (1982) *Plant Physiol. 70,* 965-970
MacDonald, F. D., Chou, Q., Buchanan, B. B. & Stitt, M. (1989) *J. Biol. Chem. 264,* 5540-5544
Neuhaus, H.E., Kruckeberg, A.L., Feil, R. & Stitt, M. (1989) *Planta 178,* 110-122
Preiss, J., Robinson, N., Spilatro, S. & McNamara, K. (1985) in *Regulation of Carbon Partitioning in Photosynthetic Tissue* (Preiss, J. & Heath, R.L., eds.), pp. 1-26, American Society of Plant Physiologists, Baltimore, Maryland
Quick, P., Neuhaus, H. E., Feil, R. & Stitt, M. (1989) *Biochim. Biophys. Acta 973,* 263-271
Rodermel, S. R., Abbott, S. N. & Bogorad, L. (1989) *Cell 55,* 673-681
Smith, A. M., Neuhaus, H. E. & Stitt, M. (1990), *Planta* in press
Stitt, M., Gerhardt, R., Kürzel, B. & Heldt, H. W. (1983) *Plant Physiol. 72,* 1139-1141
Stitt, M., Cseke, C. & Buchanan, B. B. (1984*a*) *Eur. J. Biochem. 143,* 89-93
Stitt, M., Kürzel, B. & Heldt, H. W. (1984*b*) *Plant Physiol. 75,* 554-560
Stitt, M. & Heldt, H. W. (1985*a*) *Planta 164,* 179-188
Stitt, M. & Heldt, H. W. (1985*b*) *Plant Physiol. 79,* 599-608
Stitt, M., Gerhardt, R., Wilke, I. & Heldt, H. W. (1987*a*) *Physiol. Plantarum 69,* 377-386
Stitt, M., Huber, S. C. & Kerr, P. (1987*b*) in *The Biochemistry of Plants* (Hatch, M.D. & Boardman, N.K., eds.), Vol. 10, pp. 327-407, Academic Press, London
Stitt, M. & Große, H. (1988) *J. Plant Physiol. 133,* 392-400
Stitt, M., Wilke, I., Feil, R. & Heldt, H. W. (1988) *Planta 174,* 217-220
Stitt, M. (1989*a*) *Phil. Trans. R. Soc. Lond. B 323,* 327-338
Stitt, M. (1989*b*) in *Techniques and New Developments in Photosynthesis Research* (Barber, J. & Malkin, R., eds.), Plenum Publ. Company, New York, in press
Stitt, M. & Quick, P. (1989) *Physiol. Plantarum,* in press
Walker, J. L. & Huber, S. C. (1988) *Planta 177,* 116-120
Weiner, H., Stitt, M. & Heldt, H. W. (1989) *Biochim. Biophys. Acta 893,* 13-21
Wirtz, W., Stitt, M. & Heldt, H. W. (1980) *Plant Physiol. 66,* 187-193

Chapter 32

Application of Control Analysis to the Study of Amino Acid Metabolism

RICHARD G. KNOWLES, CHRISTOPHER I. POGSON
and MARK SALTER

FOR LABORATORY scientists studying the control of metabolism, the usefulness or otherwise of theories of metabolic control is determined by the ease of their utility for the analysis of experimental data, and the insights into the control of metabolism provided by this data analysis. In this chapter we describe the application of the metabolic control theory developed by Higgins (1965), Kacser & Burns (1973) and Heinrich & Rapoport (1974) to our studies of amino acid metabolism both previous and current. We have found this theory of control analysis and modelling of the pathways under study, to be invaluable tools in the elucidation of the control structure of amino acid metabolism. The distinction between regulatory importance, regulability and control, and the way in which the regulability and control coefficients of an enzyme combine to describe its regulatory importance will be discussed.

Application to Aromatic Amino Acid Catabolism in the Liver

Previously the control of these pathways in the liver has been described solely in terms of the "rate-limiting" roles of the first enzymes of these pathways: tryptophan 2,3-dioxygenase, phenylalanine hydroxylase and tyrosine aminotransferase. By using control analysis we have shown that this simplistic view of the control structure is false (Salter *et al.*, 1986*a*). High flux control coefficients were shown to occur in the aromatic amino acid transport into the liver cells (Salter *et al.*, 1986*b*) as well as in the first enzymes of the pathways. It was also clear that this control structure was not static, but changed with changes in the expressed activities of the different enzymes or transporters. It was interesting to observe high flux control coefficients in steps such as transport, which are readily reversible, are not far from equilibrium under pathway conditions and have V_{max} values greatly in excess of the path-

Richard G. Knowles, Christopher I. Pogson and Mark Salter ● Department of Biochemical Sciences, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

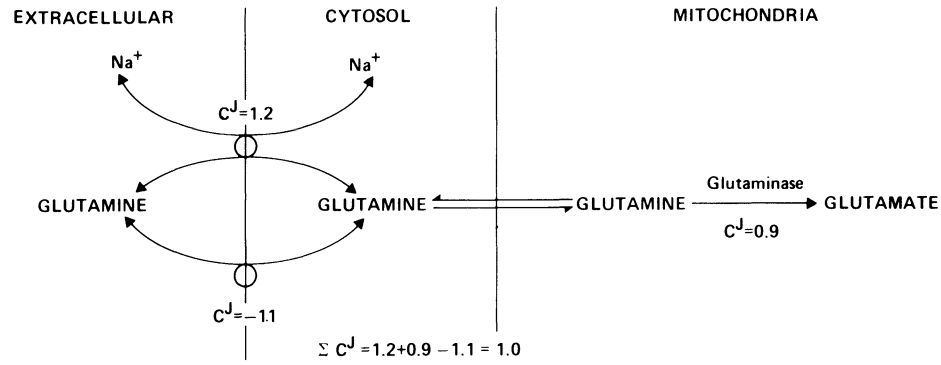


Figure 1. Glutamine metabolism in isolated liver cells.

way flux; such characteristics have previously been thought to indicate that such a step would not exert significant control over a pathway.

Application to Glutamine Catabolism in the Liver

There has been considerable debate about the relative importance of glutamine transport and glutaminase in determining the rate of glutamine catabolism in the liver (reviewed in Ochs, 1986). We have used both published and unpublished (S. Low, unpublished work) data to model glutamine transport and catabolism (see Fig. 1) and to analyse the control structure of this pathway at physiological concentrations of glutamine. Glutamine is transported across the liver cell plasma membrane by two distinct transport systems; one is a sodium-dependent concentrative transporter (system N) which catalyses net influx into the cell (Kilberg *et al.*, 1980) and the remaining transport is though to be carried out by a non-concentrative transporter which catalyses net efflux from the cell. Catabolism of glutamine to glutamate is catalysed by the mitochondrial enzyme glutaminase.

Small changes in the activities of transport or glutaminase can be made in this model in order to determine the flux control coefficients of individual steps; these are shown in Fig. 1. It is clear that a large degree of control resides in all three steps. Because of the net efflux of glutamine through the non-concentrative transporter this step has a negative control coefficient for glutamine catabolism. This large negative control coefficient permits the occurrence of greater positive control coefficients in the other two steps (the total flux-control coefficients for these two steps being 2.1). This transporter, catalysing net efflux, also acts as a safety valve preventing excessive increases in the intracellular glutamine concentration that would otherwise occur because of the low elasticity of the concentrative transporter for internal glutamine. This system of concentrative transport (catalysing net influx) and non-concentrative transport (catalysing net efflux) co-existing with a pathway of metabolism (Fig. 2) is common. The control structure of such systems, with a large degree of either negative or positive control residing in each step allows pathway flux to be very

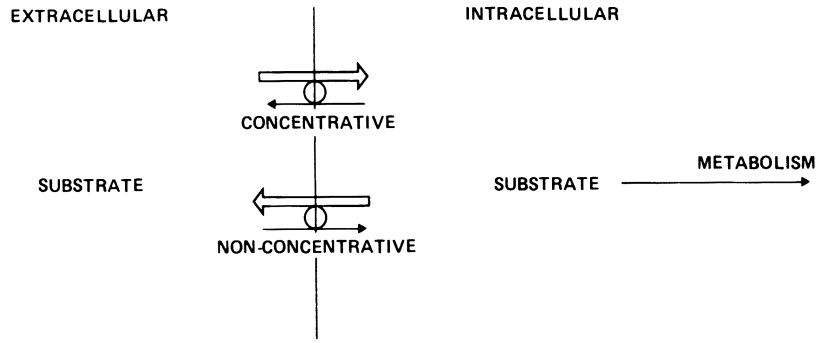


Figure 2. A general scheme for concentrative and non-concentrative transport and metabolism.

sensitive to changes in any of the steps. Our use of modelling and control analysis has therefore shown that both transport and glutaminase are important in determining the flux through this pathway; this contrasts with previous debate as to whether control resides in either transport or glutaminase. This analysis has also revealed an unexpected degree of complexity in the control structure, as a consequence of the properties of the two transporters.

Application to Tryptophan Homeostasis

There are two distinct enzymes in the body which can catalyse the irreversible breakdown of tryptophan to kynurenine, tryptophan 2,3-dioxygenase in the liver parenchymal cells and indoleamine 2,3-dioxygenase elsewhere in the body. Other pathways of tryptophan metabolism are quantitatively minor (Pogson *et al.*, 1989). Under normal conditions tryptophan 2,3-dioxygenase is thought to catalyse the majority of tryptophan catabolism, as shown in Fig. 3. Dietary tryptophan input is thought to be insensitive to the plasma tryptophan concentration, and tryptophan 2,3-dioxygenase responds essentially linearly to plasma tryptophan in this concentration range. Manipulation of activities of reaction steps in this model demonstrates that the metabolite control coefficient of tryptophan 2,3-dioxygenase

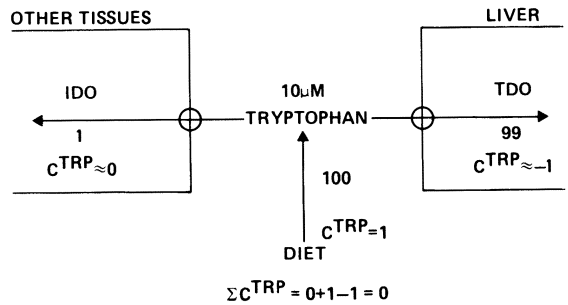


Figure 3. Tryptophan homeostasis under normal conditions

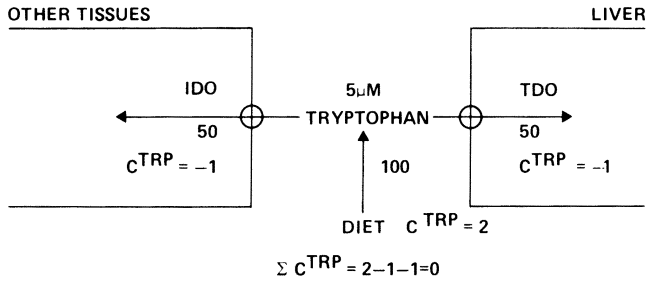


Figure 4. Tryptophan homeostasis after treatment with interferon, which induces indoleamine 2,3-dioxygenase, but not tryptophan 2,3-dioxygenase. The model assumes that indoleamine 2,3-dioxygenase is saturated with tryptophan (cf. Fig. 5).

approaches -1 whereas that of indoleamine 2,3-dioxygenase approaches 0; from the summation property (Heinrich & Rapoport, 1974; Kacser & Burns, 1979) the dietary input can be calculated to have a metabolite control coefficient of 1 (Fig. 3).

However, under certain conditions indoleamine 2,3-dioxygenase may be substantially induced. Brown *et al.* (1987) have demonstrated a 50% decrease in the plasma tryptophan concentration of cancer patients when treated with interferon γ; interferon γ is thought to induce indoleamine 2,3-dioxygenase but not tryptophan 2,3-dioxygenase. In our model (Fig. 4) we have assumed that indoleamine 2,3-dioxygenase is saturated with tryptophan and that dietary tryptophan input is unchanged; a 50% decrease in plasma tryptophan concentration implies that indoleamine 2,3-dioxygenase has been induced to a level at which it catalyses flux equal to that through tryptophan 2,3-dioxygenase. Again manipulating the activities of reaction steps in this model allows the calculation of metabolite control coefficients: under these conditions both indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase have metabolite control coefficients approaching -1, and dietary input therefore has a metabolite control coefficient of 2. In a model in which indoleamine 2,3-dioxygenase is taken to be unsaturated with tryptophan (Fig. 5), the metabolite control coefficients of indoleamine 2,3-dioxygenase, tryptophan 2,3-dioxygenase and dietary input are -0.5, -0.5 and 1 respectively.

Using this approach we have therefore obtained considerable insight into tryptophan

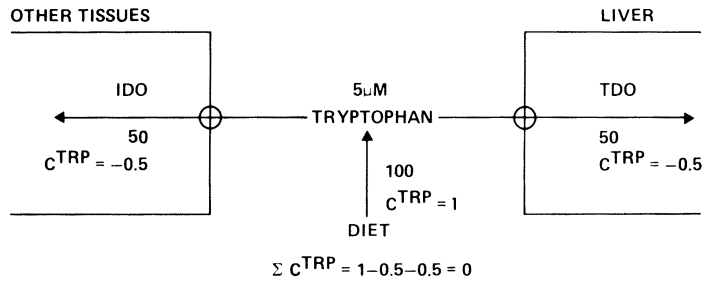


Figure 5. Tryptophan homeostasis after interferon treatment. The model differs from the one used for Fig. 4 in assuming that indoleamine 2,3-dioxygenase is saturated with tryptophan.

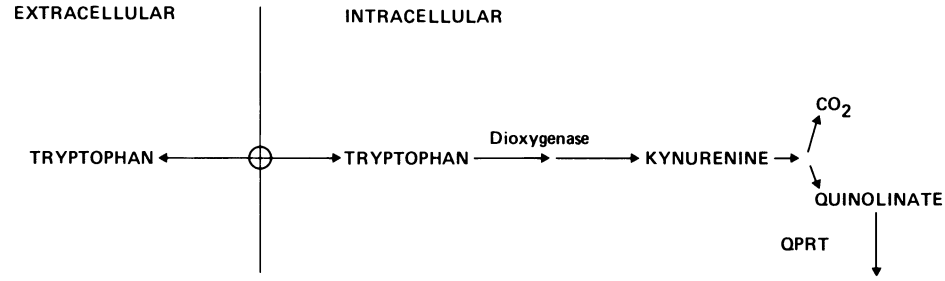


Figure 6. Metabolism of tryptophan to quinolinate in liver and brain. *Abbreviation:* QPRT, quinolate phosphoribosyltransferase

homeostasis on the basis of a limited amount of data. The values obtained by modelling tryptophan homeostasis await experimental verification using specific inhibitors of indoleamine 2,3-dioxygenase or tryptophan 2,3-dioxygenase.

Application to Quinolinate Metabolism

Quinolinate can be formed from tryptophan in the liver and the brain by the kynurenine pathway (reviewed in Stone & Connick, 1985: Fig. 6). The pathway in the liver is initiated by tryptophan 2,3-dioxygenase whereas that in the brain is initiated by indoleamine 2,3-dioxygenase. Recent work has shown quinolinate to be an exitatory agonist at the *N*-methyl-D-aspartate receptor in the brain; high concentrations of quinolinate have been demonstrated to cause neuronal cell death as a consequence of extreme excitation at this receptor. Increases in quinolinate in the brain have been suggested to be associated with exitatory and neurodegenerative diseases such as Huntington's Chorea and epilepsy; increases with aging have also been observed in rats.

Little is known about the regulation of quinolinate concentration in the brain. Quinolinate is metabolized by quinolinate phosphoribosyltransferase; this enzyme is thought to have a K_m for quinolinate which is below the physiological concentration in the brain and catalyses an irreversible reaction (Okuno *et al.*, 1988). Since the substrate elasticity will therefore be small, quinolinate phosphoribosyltransferase will have a large metabolite control coefficient for quinolinate and increases in quinolinate synthesis will result in large increases in quinolinate concentration.

Under conditions in which indoleamine 2,3-dioxygenase is not induced, it will have low metabolite control coefficients for plasma tryptophan (see above) and for intracellular tryptophan, because of the fast rate of tryptophan transport (Pogson *et al.*, 1989), and is likely to have a significant control coefficient for quinolinate synthesis in the brain. Given the low elasticity of quinolinate phosphoribosyltransferase for quinolinate, increases in indoleamine 2,3-dioxygenase activity would result in large increase in quinolinate concentration, i.e. indoleamine 2,3-dioxygenase will have a large metabolite control coefficient. Because of the high metabolite control coefficient of tryptophan 2,3-dioxygenase for plasma tryptophan (see above), and if indoleamine 2,3-dioxygenase is unsaturated with tryptophan

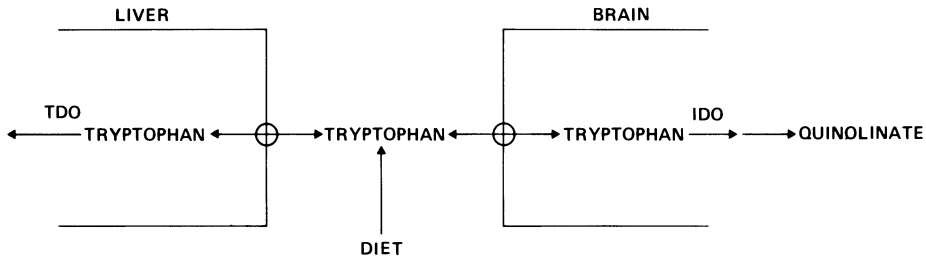


Figure 7. The role of tryptophan 2,3-dioxygenase in the control of metabolism of tryptophan to quinolinate in the brain. *Abbreviations:* TDO, tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase.

in the brain then tryptophan 2,3-dioxygenase in the liver will also have a significant (but negative) metabolite control coefficient for quinolinate in the brain (Fig. 7).

It can be seen therefore that the elucidation of this control structure has allowed us to identify sites of possible therapeutic importance.

Regulability, Control and Regulatory Importance

There has been much debate and confusion about the importance of control coefficients and their role in describing the regulatory importance of enzymes. This is largely caused by the absence of clear consistent usage of terms such as control, regulability and regulatory importance.

There would perhaps be less confusion in this field if "control" was used to denote the sensitivity of flux or metabolite concentrations to changes in the activity of steps in the pathway (i.e. control coefficients), "regulability" to denote the degree to which enzyme activity (as expressed under pathway conditions) can be changed by any effector, and the "regulatory importance" of an enzyme would be a property derived from these two factors together, and would therefore define the ability of an enzyme to change pathway flux (or the concentration of a metabolite in that pathway).

The only obvious way to quantify this characteristic of regulatory importance would be to formulate a theory combining control coefficients and regulability of enzymes. However, because the control coefficients of enzymes usually change significantly as a consequence of regulation of their activity, it would first be necessary to formulate theories describing how control coefficients change with changes in expressed enzyme activity under pathway conditions.

Another reason for the desirability of knowing how control coefficients change with changes in enzyme activity is to facilitate the accurate determination of low control coefficients by inhibitor titration. Accurate measurement of low control coefficients could be useful for the identification of therapeutic targets in a pathway since it would allow the investigator to determine what degree of enzyme inhibition (regulability) would be necessary for the required inhibition of pathway flux. The identification of the optional site(s) for therapeutic intervention would require the identification of the enzyme with the best com-

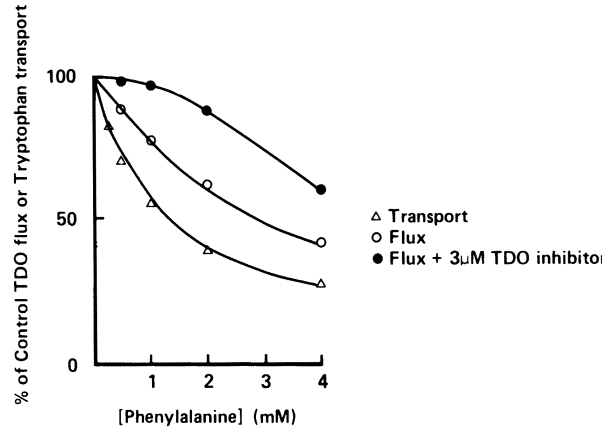


Figure 8. The inhibition of tryptophan transport and catabolic flux by phenylalanine. *Abbreviation:* TDO, tryptophan 2,3-dioxygenase.

bination of control coefficient and regulability (given that inhibitors are more easily developed for some enzymes than others).

That it is difficult to obtain accurate values of low control coefficents by inhibitor titration (without knowledge of how the control coefficient changes with enzyme activity) is shown by the following example, using the data shown in Fig. 8. This figure shows the effect of the tryptophan transport inhibitor, phenylalanine, on tryptophan catabolic flux (in the presence or absence of a tryptophan 2,3-dioxygenase inhibitor), and on tryptophan transport, in isolated rat liver cells. Calculation of flux control coefficients of transport from the data at 0.5mM phenylalanine gives values of 0.08 ± 0.08 (mean ± standard deviation) in the presence of the tryptophan 2,3-dioxygenase inhibitor and 0.42 ± 0.10 in its absence. It is clear that using the flux inhibition data at 0.5 mM phenylalanine (at which concentration transport has already been inhibited by 30%) it is virtually impossible to calculate accurately control coefficients which are below 0.1, using inhibitor titration, even when one has errors as small as those shown in Fig. 8 (standard deviations approximately 2-3% of mean). It is possible that use of data from the whole of the inhibition curve, together with knowledge of how the control coefficients change with changes in the enzyme or transporter activity, would permit far more accurate determination of low control coefficents.

Conclusions

The examples of experimental studies given above illustrate the usefulness of control analysis as a theoretical framework enabling the elucidation of the control of metabolism and allowing the postulation of new hypotheses for experimental verification or falsification. We have been surprised by how frequently such analysis gives new and unexpected insights into the control of metabolism, and suggests possible novel sites of therapeutic intervention.

We consider that control theory needs to be extended to embrace more fully the regulability of enzymes as well as their control coefficients, and to facilitate our understanding of how control coefficients change with changes in enzyme activity.

REFERENCES

Brown, R. R., Borden, E. C., Sondel, P. M., & Lee, C. M. (1987) in *Tryptophan and Serotonin Research, 1986* (Bender, D. A., Joseph, M. H., Koche, W. & Steinhart, H., eds.) pp. 19-26, Walter de Gruyter, New York

Heinrich, R & Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 107-120

Higgins, J. (1965) in *Control of Energy Metabolism* (Chance, B., Estabrook, R. K., & Williamson, J. R., eds.) pp. 13-46, Academic Press, New York

Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104

Kacser, H & Burns, J. A. (1979) *Biochem. Soc. Trans. 7,* 1149-1161

Kilberg, M. S., Handlogten, M. E. & Christensen, H. N. (1980) *J. Biol. Chem. 255,* 4011-4019

Ochs, R. S. (1986) *Trends Biochem. Sci. 11,* 235-236

Okuno, E., White, R. J., & Schwarcz, R. (1988) *J. Biochem. 103,* 1054-1059

Pogson, C. I., Knowles, R. G. & Salter, M. (1989) *Crit. Rev. Neurobiol.,* in press

Salter, M., Knowles, R. G. & Pogson, C. I. (1986*a*) *Biochem. J. 234,* 635-647

Salter, M., Knowles, R. G. & Pogson, C. I. (1986*b*) *Biochem. J. 233,* 499-506

Stone, T. W., & Connick, J. H. (1985) *Neuroscience 15,* 597-617

Chapter 33

Flux Control Coefficients of Glycinamide Ribonucleotide Transformylase for *de novo* Purine Biosynthesis

GARY K. SMITH, RICHARD G. KNOWLES, CHRISTOPHER I. POGSON, MARK SALTER, M. HANLON and R. MULLIN

THE PATHWAY of *de novo* purine synthesis in mammals is initiated from phosphoribosylpyrophosphate and incorporates carbon and nitrogen from glutamine, glycine and the one-carbon pool to form initially IMP, from which the other purines can be synthesized. This pathway has been considered to be largely regulated by purines at the first enzyme, phosphoribosylpyrophosphate amidotransferase. In order for this enzyme to be an effective site of feedback regulation it must have a substantial flux control coefficient compared to the other enzymes in the *de novo* pathway. This feedback inhibitory mechanism results in significant control residing outside the purine *de novo* pathway, in purine utilization (positive control) and the alternative pathway of purine synthesis, purine salvage (negative control). As part of a continuing anti-tumour effort we are investigating specific steps in the *de novo* purine synthesis as targets for inhibition in cancer chemotherapy.

Glycinamide ribonucleotide transformylase catalyses the third step in this ten-step pathway, the conversion of glycinamide ribonucleotide to formyl glycinamide ribonucleotide; the co-substrate for this reaction is 10-formyltetrahydropteroylpolyglutamate. Using an inhibitory analogue of its folate substrate, 5-deazaacyclotetrahydrofolate, the goal of the current study was to determine the flux control coefficient of this enzyme in the human T-cell leukaemia line MOLT-4.

Results and Discussion

The following equation, modified from Kacser & Burns (1973) and Groen *et al.* (1982), was used to determine the flux control coefficient of an irreversible enzyme (such as

Gary K. Smith, M. Hanlon and R. Mullin ● Department of Medicinal Chemistry, Burroughs Wellcome Company, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709, USA ❖ Richard G. Knowles, Christopher I. Pogson and Mark Salter ● Department of Biochemical Sciences, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

glycinamide ribonucleotide transformylase) using a competitive inhibitor:

$$C^J = -\left[\frac{I_x + K_i\left(1 + \frac{S}{K_m}\right)}{J_x}\right]\left(\frac{dJ}{dI}\right)$$

in which I_x and J_x are the intracellular inhibitor concentration and pathway flux respectively under the conditions being studied and S is the intracellular concentration of the competing substrate.

Both 5-deazaacyclotetrahydrofolate and the folate substrate are polyglutamated inside the cell to form the active species. The K_i and K_m of the hexaglutamated compounds are 0.4 μM and 3.3 μM respectively (R. Ferone, M. Hanlon, J. K. Kelley, E. W. McLean, N. K. Cohn, D. S. Duch, M. Edelstein and G. K. Smith, unpublished work). Measurements of the inhibitor concentration inside the cell showed that at steady state (24 hours exposure) it accumulated approximately 300-fold over external levels over the range of inhibitor concentrations used. The intracellular concentration of the polyglutamated form of the folate substrate in these cells was also measured and was found to be approximately 1.1 μM. *De novo* purine synthesis from 3-^{14}C-serine in MOLT-4 cells was determined after incubation for 24 hours with 0-100 nM extracellular concentrations of 5-deazaacyclotetrahydrofolate. At 100 nM 5-deazaacyclotetrahydrofolate *de novo* purine synthesis was inhibited approximately 90%. The slope of the sigmoidal curve of flux against inhibitor concentration (dJ/dI) was estimated at various points on the curve (I_x, J_x) and then used to determine flux control coefficients using the equation above. The results of these experiments can be seen in Table 1. In the approximate absence of inhibitor it is clear that the flux control coefficient is very close to zero but as the enzyme is inhibited the flux control coefficient increases to a value that is close to unity.

Although in Table 1 we have given a value of 0.01 for the flux control coefficient of glycinamide ribonucleotide transformylase in the approximate absence of inhibitor, in reality this value cannot be determined with greater accuracy than ± 0.1. This would seem to be a general problem of determining flux control coefficients by this method as with normal experimental error (in this case a standard error of the mean in the range 3-4%) a significant

Table 1. Effect of 5-deazaacyclotetrahydrofolate on the activity of glycinamide ribonucleotide transformylase (GARTF) and *de novo* purine biosynthesis in MOLT-4 cells

[5-deazaacyclotetrahydrofolate] Extracellular (nM)	Intracellular (nM)	Inhibition of Activity (%)	Inhibition of Flux (%)	C^J_{GARTF}
0	0	0	0	0.01
5	1500	75	1	0.03
10	3000	86	2	0.1
25	7500	94	29	0.7
40	12000	96	56	1.2
50	15000	97	62	1.3
100	30000	98	86	1.2

change in flux will be required to obtain an accurate slope. When the enzyme under study has a low flux control coefficient, as in this case, then substantial inhibition of the enzyme activity must occur before any significant change in the flux can be seen. For example, at 10 nM extracellular 5-deazaacyclotetrahydrofolate (3 μM, intracellular) the flux has not significantly changed but it can be calculated that at this concentration of inhibitor the enzyme will be approximately 90% inhibited. The flux control coefficient may well have significantly changed with such a change in enzyme activity but is unobservable. It may be possible to use data from the whole curve of flux inhibition to obtain greater accuracy.

It is clear from these results that not only is the absolute value of the control coefficient of a step of interest important, but also the way in which the control coefficient changes on regulation of the enzyme. In this instance a step with a very low flux control coefficient can nevertheless represent a site at which *de novo* purine synthesis can be regulated. Thus, the suitability of an enzyme as a target for therapeutic intervention is is also determined by the degree to which its activity can be modulated by an effector. This will be limited in practice by considerations such as the affinity of an available inhibitor, its distribution within the body, its pharmacokinetics and its toxicity. In this instance significant inhibition of *de novo* purine biosynthesis by 5-deazaacyclotetrahydrofolate is achieved as a result of its high enzyme affinity and high intracellular concentrations achieved after exposure for 24 hours.

Conclusions

We have shown that glycinamide ribonucleotide transformylase has a very low flux control coefficient for purine synthesis under normal conditions but that on extensive inhibition by 5-deazaacyclotetrahydrofolate this flux control coefficient increases approximately to unity. Under the conditions in which glycinamide ribonucleotide transformylase has a flux control coefficient of unity we would suggest that *de novo* flux control will no longer reside in purine utilization or purine salvage as a consequence of the effective loss of the feedback loop from purines to phosphoribosylpyrophosphate amidotransferase. This would be predicted to occur because of the substantially reduced flux control coefficient in phosphoribosylpyrophosphate amidotransferase.

Our studies also indicate that the suitability of a site for therapeutic intervention is a function both of its control coefficient and the extent of achievable inhibition (regulability).

REFERENCES

Groen, A. K., Van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, T. P. M. & Tager, J. M. (1982) in *Metabolic Compartmentation* (Sies, H., ed.) pp 9-37, Academic Press, London

Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104

Chapter 34

Molecular Adaptation in the Lactose Operon

ANTONY M. DEAN

MODERN studies of molecular evolution are dominated by sequence comparisons among homologous genes of present day species. This approach, which enables the construction of gene phylogenies that reflect the historical relations among species, has been crowned with the spectacular discovery of a new kingdom — the archaebacteria (Woese, 1987).

Natural selection plays less role in discussions of molecular evolution than might be imagined, partly because gene function is largely irrelevant to this comparative anatomy of macromolecules, and partly because it need rarely be invoked. An analysis of virtually any gene phylogeny will show that the rates of sequence divergence have been remarkably constant for millions of years in the many diverse lineages (Wilson *et al.*, 1977). This clock-like behaviour need only be regarded as the consequence of mutation bringing new alleles into the gene pools, and the allele frequencies changing as a result of the random sampling of alleles at each generation. Thus, chance alone is responsible for divergence in this, the neutral theory of molecular evolution (Kimura, 1983).

The claim that most of the molecular differences between species are the result of chance in no way diminishes the importance of natural selection. Natural selection is the only mechanism by which species adapt to changed environments, and a failure to adapt may result in extinction. Not surprisingly, the comparative approach has also been used to study natural selection. But here it has met with considerably less success because the relations between gene function, metabolism and adaptation are never explicitly addressed. At best, the comparisons yield *a posteriori* explanations for certain functional characteristics that might be adaptive.

In principle, an experimental approach can determine these relations. Unfortunately, past attempts to unite biochemistry, metabolism, physiology and natural selection have foundered because the experimental systems were too complicated, too ill-defined, or simply lacking the necessary experimental controls for a thorough and convincing analysis. There is not a single paper in the literature in which the sign and magnitude of a selection coefficient can be predicted for a given difference in enzyme kinetics.

This chapter describes studies on the lactose operon of *Escherichia coli* (Dean *et al.*,

Antony M. Dean ● Department of Biochemistry, University of California, Berkeley, California 94720, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

1986, 1988*ab;* Dykhuizen *et al.*, 1987; Dean, 1989). A simple linear pathway, in a simple prokaryote, in the simple environment of a laboratory continuous culture device is a modest, yet practical, way to start to untangle the relations between enzyme activity, metabolism and natural selection. The result is an ability to determine the direction and magnitude of natural selection for any given difference in enzyme kinetics. Moreover, speculation on the future course of molecular adaptation and on past selection pressures will be gamefully entertained.

Measuring Fitness

Natural selection occurs when an allele spreads, at the expense of others, through a population by endowing its bearer with higher relative fitness. So what, if anything, is fitness? For our purposes, fitness is the growth rate of a strain of *E. coli* and relative fitness is nothing more than a ratio of growth rates. A relative fitness of less than one means that the strain will be eliminated by natural selection and a relative fitness of greater than one means that it will take over the entire population, again by natural selection. A relative fitness of one means that there is no natural selection and evolution can only proceed by chance changes in the frequencies of the strains.

The best method for estimating relative fitness in *E. coli* is a mixed culture of two strains in a chemostat where differences in relative fitness as small as 0.5% can be reproducibly detected (Dykhuizen & Hartl, 1983). Unlike batch growth, the chemostat maintains a culture in a permanent exponential growth phase by a slow, but constant, addition of fresh medium into the growth chamber. An overflow siphon removes the cells and spent medium so that a constant volume is maintained. The medium is composed of minimal salts, and the strains compete for lactose which is both the sole source of carbon and energy and the only nutrient limiting the growth-rates. Indeed, consumption by the culture reduces the concentration of incoming lactose to such a low level that inducible lactose operons become repressed. Consequently, competition experiments are conducted between constitutive operons, or between inducible operons which are induced by the addition of a small quantity of a non-metabolizable inducer (Dykhuizen & Davies, 1980; Dean *et al.*, 1988*a*). The expression of the operon is entirely under the control of the experimenter and so the evolution of gene regulation will not be discussed.

The relative fitness conferred by a lactose operon is determined as follows. First, the lactose operon is transduced into a small *lac* deletion of an otherwise prototrophic K12 genetic background. This step eliminates any fitness differences conferred by unrelated genetic backgrounds. Second, the strain is placed in competition with TD1, which carries a K12 lactose operon in the same genetic background, and which is also resistant to the bacteriophage T5. The progress of the competition is periodically monitored by estimating the proportion of the culture that is T5 resistant.

The difference between the exponential growth rates of the two competing strains is determined from

$$\ln\left[R_t/(1 - R_t)\right] = \ln\left[R_0/(1 - R_0)\right] + (w_R - w_S)t \qquad (1)$$

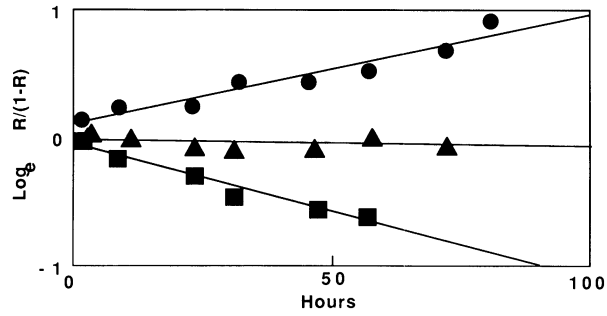


Figure 1. Results from a typical selection experiment. Glucose competition between TD1 (resistant) and TD9 (▲). Lactose competition between TD1 (resistant) and TD9 (●), and between TD1 and TD9 (resistant) (■).

where R_t is the proportion of the culture that is T5 resistant at time t, and w_R and w_S are the exponential growth rates (fitnesses) of the resistant and sensitive strains respectively. The slope of the plot of the log density ratio of the two strains against time is equal to the difference in fitness, $(w_R - w_S)$. The growth rate of the culture is equal to the fractional rate of replacement of the culture by fresh medium, Δ. Hence the relative fitness of the resistant with respect to the sensitive strain is approximately as follows:

$$w_R/w_S = 1 + (w_R - w_S)\Delta \qquad (2)$$

For the purposes of this chapter, relative fitness is always expressed with respect to the fully induced K12 operon of TD1 whose fitness, as it turns out, is exactly equivalent to that of the constitutive K12 operon of strain TD2.

Some typical results are illustrated in Fig. 1 (Dean, 1989). A set of competition experiments were conducted between TD1 and TD9, which carries an operon from a natural isolate of *E. coli*. The absence of detectable levels of selection during competition for glucose demonstrates that only differences encoded by the lactose operons could cause selection during competition for lactose. Two lactose competition experiments were conducted, in the first of which TD1 was resistant to T5, and in the second of which TD9 was resistant. The symmetrical selective response demonstrates that T5 resistance confers no detectable selective effect during competition for lactose. The relative fitness of TD9 is 0.967 which means that natural selection will eventually eliminate it from the chemostat population. The problem, then, is to understand this selection in terms of the biochemistry of lactose metabolism.

Fun with Flux

The chemostat competition experiments demonstrate that different lactose operons confer different fitnesses. Presumably, fitness is partially determined by the rate at which lactose is metabolized and this, in turn, is partially determined by the kinetic differences among the permeases and β-galactosidases encoded by the lactose operons. If fitness is a function of the flux, then a model of flux must be constructed.

The lactose flux has three steps of interest. First, lactose enters the periplasmic space through the porin pores of the outer cell wall. Porins are not specific carriers of substrates (Nikaido & Vaara, 1987) and the flux across the outer cell wall can be adequately accounted for by Fick's law of passive diffusion

$$J_{wall} = D(L_m + L_p) \tag{3}$$

in which J_{wall} is the flux, L_m and L_p are the concentrations of lactose in the external medium and the periplasmic space respectively, and D is the rate constant of this diffusion process.

Second, active uptake by the *lacY* encoded permease transports the lactose from the periplasm into the cytosol through the otherwise impermeable cell membrane. The exit of galactosides appears to be associated with a very large Michaelis constant (Winkler & Wilson, 1966) so that an appropriate model for lactose uptake is as follows:

$$J_{permease} = \frac{V_{permease}(L_p - L_i/K_{eqm})}{K_t + L_p} \tag{4}$$

where $J_{permease}$ is the net lactose flux across the membrane, $V_{permease}$ and K_t are the maximum rate of uptake and the apparent Michaelis constant respectively, L_i is the concentration of lactose in the cytosol and K_{eqm} is the apparent equilibrium constant.

Third, lactose is irreversibly hydrolysed by β-galactosidase to glucose and galactose, which eventually enter central metabolism as glucose 6-phosphate. The kinetics of β-galactosidase are complicated by various side reactions of which the synthesis and irreversible hydrolysis of allolactose, the true inducer of the lactose operon, are by far the most significant (Huber *et al.*, 1976). However, data from kinetic studies, in which the allolactose side reaction is allowed to reach a quasi-steady state, fit the Michaelis-Menten form very well (Dean, 1989) and so

$$J_{\beta\text{-galactosidase}} = \frac{V_{\beta\text{-galactosidase}} L_i}{K_m + L_i} \tag{5}$$

The fluxes across each of these three steps are equal at steady state, so $J_{wall} = J_{permease} = J_{\beta\text{-galactosidase}}$ and eqns. (3-5) can be solved to yield

$$J = \frac{L_m}{\frac{1}{D} + \frac{K_t + L_p}{V_{permease}} + \frac{K_m + L_i}{K_{eqm} V_{\beta\text{-galactosidase}}}} \tag{6}$$

where J is the steady state flux, and the lactose metabolite pools L_p and L_i are functions of the concentration of environmental lactose (L_m) and the kinetic parameters at each step. Metabolite pools distal to β-galactosidase are not expected to influence this flux because the hydrolysis of lactose is irreversible, and none of the steps are known to be influenced by allosteric effectors. One may hope that eqn. (6) is an adequate model of the lactose flux.

From Flux to Fitness

The rate at which lactose changes during growth in a chemostat is described by

$$\frac{dL_m}{dt} = \Delta(L_0 - L_m) - \frac{wN}{Y} \tag{7}$$

where L_m is the concentration of lactose in the growth chamber, L_0 is the concentration of lactose in the fresh medium dripping into the growth chamber, Y is the yield coefficient of cells per gram of lactose consumed and w is fitness (Monod, 1942). Rewriting the equation to emphasize the flux, J, in the process gives

$$\frac{dL_m}{dt} = \Delta(L_0 - L_m) - JN \tag{8}$$

Hence $J = w/Y$, which can be rewritten using eqn. (6) as follows:

$$w = YJ = \frac{YL_m}{\frac{1}{D} + \frac{K_t + L_p}{V_{permease}} + \frac{K_m + L_i}{K_{eqm} V_{\beta\text{-galactosidase}}}} \tag{9}$$

The yield coefficient is virtually constant over the range of growth rates of interest, and therefore flux is predicted to be directly proportional to fitness.

The intense competition for lactose in the chemostat ensures that L_p and L_i will be much smaller than the respective Michaelis constants (Dean, 1989). Hence, the relative fitness of a strain X with respect to that of the common competitor TD1 is predicted to be equal to the ratio of the fluxes, and

$$\frac{w_R}{w_S} = \frac{J_X}{J_{TD1}} = \frac{\frac{1}{D_{TD1}} + \frac{K_{t,\,TD1}}{V_{permease,\,TD1}} + \frac{K_{m,\,TD1}}{K_{eqm} V_{\beta\text{-galactosidase},\,TD1}}}{\frac{1}{D_X} + \frac{K_{t,\,X}}{V_{permease,\,X}} + \frac{K_{m,\,X}}{K_{eqm} V_{\beta\text{-galactosidase},\,X}}} \tag{10}$$

The kinetic parameters and relative fitnesses were determined for the permeases and β-galactosidases of various operons. In total, five mutant β-galactosidases (Dean *et al.*, 1985), four lactase alleles from the ebg locus (Hall, 1984), six operons from world-wide natural isolates of *E. coli* in competition with TD1 (Dean, 1989) and the fitness effects of modulating expression of an inducible operon in competition with a constitutive operon (Dykhuizen & Davies, 1980; Dykhuizen *et al.*, 1987) have been investigated. The uptake of radiolabelled lactose by intact cells provided parameter estimates for both the diffusion step and the permease step, whilst the *in vitro* hydrolysis of lactose by β-galactosidase was assayed by coupling the production of galactose to the reduction of NADP by galactose dehydrogenase. The estimate of K_{eqm} = 442 was obtained as an average of estimates found in the literature (Winkler & Wilson, 1976; Wright *et al.*, 1981). Thus, all the data necessary to estimate the relative fluxes have been obtained, together with the observed relative fitnesses of each strain.

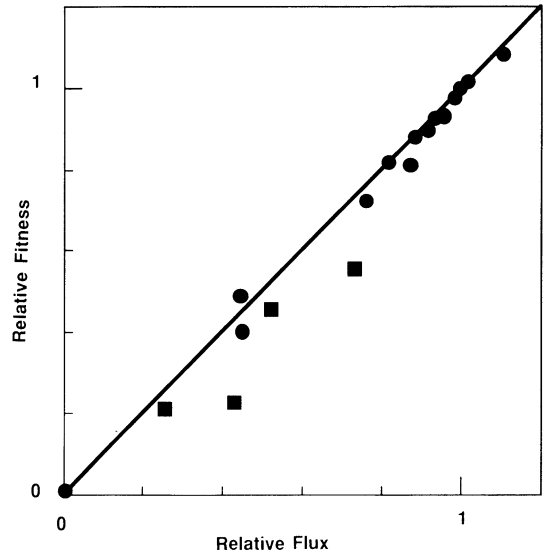


Figure 2. The relation between fitness and flux for variants of the lactose operon, either the permease, the β-galactosidase or both (●), and for variant alleles of *ebgA* (■, Hall, 1984).

As predicted, relative fitness, w_X/w_{TD1}, is directly proportional to relative flux, J_X/J_{TD1} (Fig. 2). Relative fitness would not necessarily be proportional to the model of relative flux if a step in the pathway had been omitted. Had this been the case, the flux control coefficients of the permease and β-galactosidase would be inflated by a common factor compared to their fitness control coefficients. But Table 1 shows a good agreement between the control coefficients predicted from the kinetics studies of TD1 and those experimentally determined from the many competition experiments. Moreover, the sum of the control coefficients is not significantly different from unity. Hence, the flux summation theorem of Kacser & Burns (1973, 1981) is confirmed and eqn. (10) can be rewritten as follows:

$$\frac{w_R}{w_S} = \frac{J_X}{J_{TD1}} = \frac{1}{\frac{C^J_{diffusion}}{k_{diffusion}} + \frac{C^J_{permease}}{k_{permease}} + \frac{C^J_{\beta\text{-galactosidase}}}{k_{\beta\text{-galactosidase}}}} \tag{11}$$

where $C^J_{diffusion}$, $C^J_{permease}$ and $C^J_{\beta\text{-galactosidase}}$ are the flux control coefficients, and the three k values are the first-order rate constants of the variants expressed as a proportion of those of TD1. Note that $k_{diffusion} = 1$ for all strains because the lactose operon encodes no structural component of the cell wall.

Table 1. TD1 Control Coefficients on Limiting Lactose

	Cell wall	Permease	β-Galactosidase	Sum
Predicted (kinetics)	0.819	0.178	0.003	1
Observed (fitness)	0.882 ± 0.085	0.158 ± 0.029	0.003 ± 0.0003	1.04 ± 0.09

In a sense, eqns. (10-11) represent a sort of missing link between molecular evolution, as represented by relative fitness on the left, and molecular biology, as represented by relative flux on the right. Relative fitness is completely defined in terms of the kinetic parameters of the lactose flux. Consequently, this equation can be used to predict the magnitude and direction of selection of a previously untested lactose operon solely from a knowledge of the kinetics of its enzymes.

The Control of Natural Selection ...

In general, the fitness of one allele with respect to another can be defined as follows:

$$w_R/w_S = 1 + \delta E \cdot C_E^M \cdot C_M^W \tag{12}$$

where w_R and w_S are the fitnesses conferred by the alleles, δE is the change in enzyme activity, C_E^M is the conventional control coefficient with respect to some metabolic parameter M, and C_M^W is the control coefficient of fitness with respect to M (Dean & Dykhuizen, 1990). In our case $M = C_M^W = 1$ so eqn. (12) reduces to

$$w_R/w_S = 1 + \delta E \cdot C_E^M \tag{13}$$

We can use this equation to predict the future course of evolution in the chemostat for each step in the lactose flux. Increases in β-galactosidase activity are unlikely to have a significant impact on the fitness because the flux control coefficient is so small (Dean *et al.*, 1987). Rather, natural selection will target mutations in the cell wall and in the permease where large control coefficients allow increased activities be manifest as gains in fitness. As selection proceeds to increase cell wall permeability and permease activity, so the control coefficients of these steps will become smaller and smaller. Meanwhile, the flux summation theorem ensures that the control coefficient of the β-galactosidase will become larger and larger. Hence mutants of β-galactosidase that were previously hidden are now exposed to natural selection (Dean, 1989).

Fig. 3 shows the expected distribution of control in TD1 as a function of the concentration of environmental lactose. Again, natural selection is unlikely to target the β-galactosidase because the flux control coefficient remains small regardless of the concentration of lactose. In contrast, selection targets the cell wall only at low concentrations of lactose where the first order constant for the diffusion step (D) is rather smaller than that of the permease (the ratio $V_{permease}/K_t$). At high concentrations of lactose, all the control shifts to the permease which becomes saturated, rate limiting and subjected to intense natural selection.

The initial adaptation of the cell wall requires low concentrations of lactose in a chemostat. This fits our intuitive notion that the more available something is, the less competition there is, and hence the less effective natural selection is. Much evolutionary speculation depends on this notion of scarcity, competition and selection intensity. But as

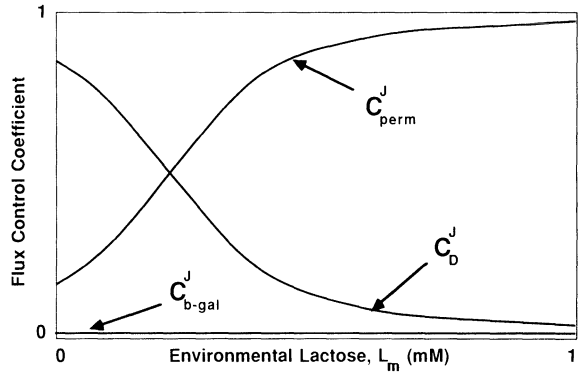


Figure 3. The distribution of flux control in the fully induced K12 lactose operon as a function of the concentration of environmental lactose. The stimulated control coefficients were obtained from the elasticities estimated using kinetics parameters obtained for TD1.

the abundance of lactose increases the control shifts to the permease which becomes increasingly exposed to natural selection. This demonstrates that there is no *a priori* reason to assume that natural selection is more intense when resources are scarce (Dean, 1989).

... *and The Natural Selection of Control*

Why is most of the control housed in the cell wall and in the permease but not in the β-galactosidase? There is nothing in the simple metabolic model that suggests why a particular distribution of control should have been favoured over any other. But in habitats other than the chemostat, the relation between fitness and flux might be rather different.

At low concentrations of lactose, most of the control is located in the cell wall. Elimination of the cell wall might increase the flux, but the cells would now be highly susceptible, for instance, to osmotic shock. Lactose diffuses from the external environment into the periplasmic space through proteinaceous channels (porins) of the outer cell wall. Expression of the *OmpF* encoded porins, which are of greater diameter, will increase the flux. However, expression of *OmpF* is repressed at 37°C in media of relatively high osmotic strength. Such are the conditions in the chemostat and also the mammalian gut, where the response may be an adaptation to prevent bile salts from entering the periplasmic space and wreaking havoc at the cell membrane (Nikaido & Vaara, 1987). These additional selective forces may prevent increases in cell wall permeability at low concentrations of lactose. Of course, these considerations are irrelevant at high concentrations of lactose where the cell wall will have a negligible control coefficient.

Some control is always present at the permease. But cells starving on lactose and constitutively expressing the permease are subject to death by a collapse in the proton motive force caused by a sudden excess of lactose (Dykhuizen & Hartl, 1978; Wilson *et al.*, 1981; Ghazi *et al.*, 1983). This selection may prevent increases permease activity if, in natural environments, *E. coli* is alternately subjected to lactose starvation and inundation (Dykhuizen *et al.*, 1987). With the first two steps subject to alternate selective forces that prevent increases in activity, gains in fitness can only be achieved at the third step. One might reasonably speculate that the low control coefficient of the β-galactosidase is the result

of selection for an increased flux, and that natural selection may have been so efficient that evolutionary improvement of the β-galactosidase is no longer possible (Hartl *et al.*, 1985; Dean *et al.*, 1986).

Conclusion

Experimental evolution combines with theories of metabolism to provide a new and powerful means of studying molecular adaptation. Insights into the molecular mechanisms of natural selection have been gained that are unobtainable from sequence comparisons. For instance, the intensity of selection may have little to do with the abundance of a resource. And there is no guarantee that the kinetic differences among homologous enzymes are adaptive because the control coefficient is equally important in determining fitness. Indeed, the β-galactosidase may be typical of a class of enzymes for which natural selection has been so effective at reducing control coefficients that even substantial activity changes have insignificant fitness effects. In such cases, the result of natural selection may be a mode of evolution that proceeds by the chance sampling of alleles from generation to generation (Hartl *et al.*, 1985). Moreover, the ability to quantify metabolism together with methods to study fitness has opened the way to predicting the future course of molecular evolution. After all, eqn. (12) suggests that natural selection can, in principle, be described for any underlying biochemical process, not just the lactose flux of *Escherichia coli*.

Acknowledgements: This work was supported by grants from the National Institutes of Health to Daniel E. Koshland, Jr., whose kindness, patience and support I greatly appreciate. I also wish to thank Bruce Morimoto, whose helpful criticisms and suggestions so markedly improved this manuscript.

REFERENCES

Dean, A. M. (1989) *Genetics,* in press
Dean, A. M. & Dykhuizen, D. E. (1990) in *Principles of Metabolic Control,* (Westerhoff, H. V., ed.) Plenum, New York
Dean, A. M., Dykhuizen, D. E. & Hartl, D. L. (1986) *Genetical Research 48,* 1-8
Dean, A. M., Dykhuizen, D. E. & Hartl, D. L. (1988*a*) *Mol. Biol. Evol. 5,* 469-485
Dean, A. M., Dykhuizen, D. E. & Hartl, D. L. (1988*b*) in *Proceedings of the Second International Conference on Quantitative Genetics* (Weir, B. S., Eisen, E. J., Goodman, M. M. & Namkoong, G., eds.), pp. 536-548, Sinauer Associates Inc., Sunderland, Massachusetts
Dykhuizen, D. E. & Hartl, D. L. (1978) *J. Bacteriol. 135,* 876-882
Dykhuizen, D. E. & Hartl, D. L. (1983) *Microbiol. Reviews 47,* 1050-1069
Dykhuizen, D. E., Dean, A. M. & Hartl, D. L. (1987) *Genetics 114,* 25-31
Ghazi, A., Therisod, H. & Shechter, E. (1983) *J. Bacteriol. 154,* 92-103
Hall, B. G. (1984) in *Microorganisms as Model Systems for Studying Evolution* (Mortlock, R.P., ed.), Plenum, New York
Hartl, D. L., Dykhuizen, E.D. & Dean, A. M. (1985) *Genetics 111,* 655-674
Huber, R. E., Kurz, G. & Wallenfels, K. (1976) *Biochemistry 15,* 1994-2001
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Kacser, H. & Burns, J. A. (1981) *Genetics 97,* 639-666

Kimura, M. (1983) *The Neutral Theory of Molecular Evolution,* Cambridge University Press, New York

Monod, J. (1942) *Recherches sur la Croissance des Cultures Bactériennes,* Hermann, Paris

Nikaido, H. & Vaara, M. (1987) in *Escherichia coli and Salmonella typhimurium,* Vol. 1, (Neidhart, F. C., ed.), pp. 7-22, American Soc. Microbiol., Washington D.C.

Wilson, A. C., Carlson, S. S. & White, T. J. (1977) *Ann. Rev. Biochem. 46,* 573-639

Wilson, D. M., Putzrath, R. M. & Wilson, T. H. (1981) *Biochim. Biophys. Acta 649,* 377-384

Winkler, H. H. & Wilson, T. H. (1966) *J. Biol. Chem. 10,* 2200-2211

Woese, C. R. (1987) *Microbiol. Rev. 51,* 221-271

Wright, J. K., Reide, I. & Overath, P. (1981) *Biochemistry 20,* 6404-6415

Chapter 35

On the Control of Gene Expression

HANS V. WESTERHOFF, JOHANNA G. KOSTER,
MARIELLE VAN WORKUM and KENNETH E. RUDD

THE CONTROL THEORY of metabolic pathways has become fairly complete (reviewed in Kacser & Porteous, 1987; Kell & Westerhoff, 1986; Westerhoff & Van Dam, 1987; Westerhoff, 1989*ab;* see also numerous chapters in this book, especially Chapter 3 by Porteous). However, it discusses control at a single level, i.e., that of metabolic pathways, without taking the variability of gene expression explicitly into account. In metabolic control theory, protein concentrations can only be reset by interventions. This type of control analysis is useful for understanding principles of metabolic control, and for understanding metabolic changes that are too quick or in too limited a metabolic system to involve changes in gene expression. Because variations in gene expression are ubiquitous and clearly implicated in metabolic regulation, it is of interest to see how the principles of metabolic control change if variable gene expression is taken into account. In this chapter we present a first attempt to extend metabolic control theory to variable gene expression. We also discuss two experimental situations in which we have analysed such control and make explicit where regulation differs from control.

Hierarchical Control Structures

Not all biochemical pathways are directly involved in the conversion of metabolic substrates and products. Some pathways are specialized in regulation of metabolism. An example is the modulation of the activity of glycogen phosphorylase by the activities of phosphorylase kinase and phosphatase. Fig. 1 gives an example where the concentration of a metabolic modifier (such as ATP) is controlled by two enzymes not otherwise involved in the pathway.

In such cases it can be useful to subdivide the overall metabolic network into two hierarchical levels. One of these corresponds to the usual pathways converting metabolites,

Hans V. Westerhoff and Marielle van Workum ● Division of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands ❖ Johanna G. Koster ● Hubrecht Laboratory, Utrecht, The Netherlands ❖ Kenneth E. Rudd ● Division of Bacterial Products, USFDA, Bethesda, Maryland, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

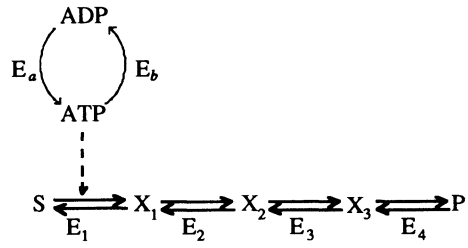


Figure 1. Example of a hierarchical control system. A metabolic pathway converts S to P. The activity of the first enzyme is affected by the concentration ratio of [ATP]/[ADP], which is increased by enzyme E_a and decreased by enzyme E_b. Control coefficients within the metabolic pathway will be indicated in the text by C, whereas control coefficients within the regulatory pathway will be indicated by Γ.

the other level houses the conversion reactions of the metabolic modifier or of the phosphorylation dephosphorylation reactions of the enzyme. As usual [review: Westerhoff & Van Dam, 1987; see also eqns. (5-6) below], control coefficients are defined as the change in the logarithm of a metabolic variable divided by the change in the logarithm of a parameter, when the latter is the only parameter that is changed. The latter change is in principle infinitesimal. Transitions between steady states are considered. Small changes in the natural logarithm of a property are equivalent to small relative changes.

Writing C for the control coefficients of the n_m enzymes in the metabolic pathway in Fig. 1, and Γ for the control coefficients by the two enzymes (E_a and E_b) in the regulatory pathway, we can express the following summation laws:

$$\sum_{i=1}^{n_m} C_i^J + \sum_{j=a,b} \Gamma_j^J = 1 \tag{1}$$

$$\sum_{i=1}^{n_m} C_i^J = 1 \tag{2}$$

$$\Gamma_a^{ATP/ADP} + \Gamma_b^{ATP/ADP} = \sum_{j=a,b} \Gamma_j^{ATP/ADP} = 0 \tag{3}$$

$$\sum_{j=a,b} \Gamma_j^J = C_1^J \cdot \varepsilon_{ATP/ADP}^1 \sum_{j=a,b} \Gamma_j^{ATP/ADP} = 0 \tag{4}$$

In words, the usual flux control summation theorem (Kacser & Burns, 1973) remains valid, independently of whether one sums over all the enzymes [(i.e. both the n_m enzymes in the metabolic pathway and the two enzymes E_a and E_b that control the [ATP]/[ADP] ratio: eqn. (1)] or only over the n_m enzymes in the metabolic pathway [eqn. (2)]. This is because the total control of the enzymes E_a and E_b on the flux through the metabolic pathway runs through [ATP]/[ADP] [eqn. (4)] and the sum of their control coefficients with respect to that concentration is zero [eqn. (3)] (cf. Heinrich *et al.*, 1977).

The regulatory pathway (in Fig. 1 merely consisting of the enzymes E_a and E_b) is subject to the usual laws of metabolic control, including the connectivity laws (cf. Kacser & Burns, 1973; Westerhoff & Chen, 1984).

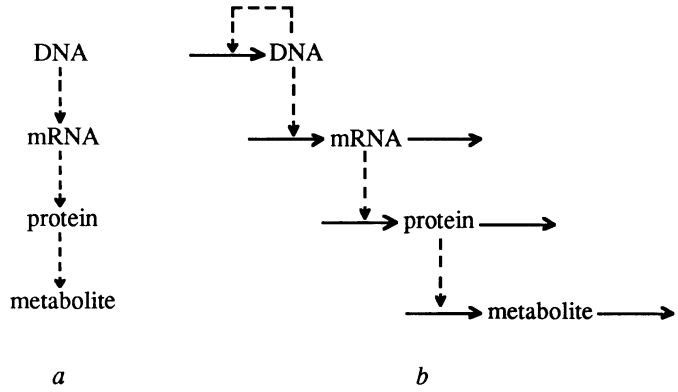


Figure 2. Dictatorial control of metabolite concentrations by gene expression. *a*, the "pathway" of control. In *b* it is stressed that this is not a metabolic pathway. There is a hierarchy of control. At each level, there is metabolism (of DNA, RNA, protein, or metabolites respectively), but the upper levels have a definite control over the lower levels. Here the extreme of a "dictatorial" hierarchy is shown, where lower levels do not affect higher levels. (Fig. 4 below illustrates a more "democratic" hierarchy.)

Variable Gene Expression: the Case of a Dictatorial Hierarchy of Control

In the existing metabolic control analysis, enzyme concentrations [or, more strictly speaking, enzyme activities (Heinrich *et al.*, 1977)] are treated as parameters, i.e. they are assumed to be constant unless "reset" by some event outside the system under study. Such resetting of these parameters has been done by use of inhibitors, or by genetic means, for instance by comparing various heterokaryons of *Neurospora crassa* (Flint *et al.*, 1981), by comparing mutants (Dean *et al.*, 1986), or by causing a gene to be expressed from a plasmid (Walsh & Koshland, 1984). The measured relative change in flux, divided by the measured change in enzyme activity, was then used to obtain the flux control coefficient by the manipulated enzyme. Thus the enzyme concentration was viewed as a controller.

From a different point of view, an enzyme concentration is a *variable* that is controlled by other parameters: it is determined by the relative activities of RNA polymerase, RNA processing enzymes, RNAases, the protein synthesizing machinery, protein processing enzymes and proteases, as well as by the gene dose and the concentration of transcriptional and translational regulators. The scheme given in Fig. 2*a* resembles that of a metabolic pathway, but differs from it in the sense that DNA is not a precursor of mRNA, mRNA is not a precursor of the enzyme, just as the enzyme is not the precursor of the product of the reaction it catalyses. Rather, mRNA functions as an activator of the protein synthesis catalysed by the ribosomal system (Fig. 2*b*).

For the moment we shall limit the discussion to the case in which the low-molecular-mass substrates, cofactors and activators for RNA and protein synthesis are in saturating or constant supply. Fig. 2*b* emphasizes that the control of gene expression involves a hierarchy. The horizontal levels correspond to metabolic pathways, one for the metabolism of

each mRNA, one for the metabolism of each enzyme and, finally, one for the metabolic pathway catalysed by the enzymes.

In Fig. 3, four enzyme complexes are involved in RNA metabolism: RNA polymerase, the spliceosome, an RNA translocase (which transports RNA from the nucleus to the cytoplasm) and RNAase. In principle, the concentrations of these enzymes are controlled by yet another level in the hierarchy. For the present discussion however, we shall take these concentrations as parameters. This allows us to define the control coefficients for the flux of RNA synthesis with respect to each of these enzymes, e.g. for the control of RNA synthetic flux J by RNA polymerase:

$$C^{J}_{\text{polymerase}} = \frac{dJ/J}{d[\text{polymerase}]/[\text{polymerase}]} = \frac{d\ln J}{d\ln[\text{polymerase}]} \tag{5}$$

Similarly, there are concentration-control coefficients for these enzymes with respect to, e.g., the concentration of mature RNA:

$$C^{[\text{RNA}]}_{\text{transcription}} = C^{[\text{RNA}]}_{\text{polymerase}} = \frac{d[\text{RNA}]/[\text{RNA}]}{d[\text{polymerase}]/[\text{polymerase}]} = \frac{d\ln[\text{RNA}]}{d\ln[\text{polymerase}]} \tag{6}$$

The dependence of the RNAase activity, v_{RNAase}, on the RNA concentration is characterized by the corresponding elasticity coefficient:

$$\varepsilon^{\text{RNAase}}_{[\text{RNA}]} = \frac{dv_{\text{RNAase}}/v_{\text{RNAase}}}{d[\text{RNA}]/[\text{RNA}]} = \frac{d\ln v_{\text{RNAase}}}{d\ln[\text{RNA}]} \tag{7}$$

Whenever the RNA metabolism is at steady state, the usual theorems hold. For instance, the sum of the control coefficients with respect to the concentration of the mRNA equals zero:

$$C^{\text{mRNA}}_{\text{polymerase}} + C^{\text{mRNA}}_{\text{splicing}} + C^{\text{mRNA}}_{\text{translocase}} + C^{\text{mRNA}}_{\text{RNAase}} = 0 \tag{8}$$

and the flux connectivity theorem with respect to the mRNA concentration reads:

$$C^{J}_{\text{polymerase}} \cdot \varepsilon^{\text{polymerase}}_{\text{mRNA}} + C^{J}_{\text{splicing}} \cdot \varepsilon^{\text{splicing}}_{\text{mRNA}} + C^{J}_{\text{translocase}} \cdot \varepsilon^{\text{translocase}}_{\text{mRNA}} + C^{J}_{\text{RNAase}} \cdot \varepsilon^{\text{RNAase}}_{\text{mRNA}} = 0 \tag{9}$$

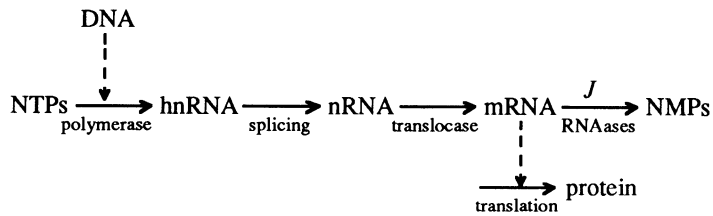


Figure 3. One of the levels of Fig. 2; the level of RNA metabolism. DNA transcription by RNA polymerase gives rise to heterogeneous nuclear RNA (hnRNA), which is processed (by the spliceosome) to yield nRNA, which is then transported across the nuclear membrane by a translocase to yield cytosolic mRNA, which may be degraded by RNAases. The remaining mRNA drives translation. Of course, this picture is still a simplification of actual RNA metabolism.

It should be noted that many of the elasticity coefficients in this last equation may equal zero. For instance, it is not likely that the cytosolic mRNA concentration has a direct influence on eukaryotic transcription. It is more orthodox to assume the mRNA concentration to affect the level of a metabolite, which then feed-back represses transcription. However, in the present section of this paper, we limit the discussion to the case where metabolite concentrations do not affect the higher levels in the hierarchy.

A theorem that is special for the hierarchical systems of gene expression, states that the sum of the control coefficients with respect to a flux or concentration, over all enzymes at a higher hierarchical level equals zero. We elaborate on this for the controls exerted by the n_R (= 4 here) enzymes involved in RNA metabolism over the rate of synthesis of a protein and over the concentration of that protein:

$$\sum_{i=1}^{n_R} \Gamma_i^{J_{protein}} = 0 \tag{10}$$

$$\sum_{i=1}^{n_R} \Gamma_i^{protein} = 0 \tag{11}$$

As in the case of hierarchies in ordinary metabolism (see above), eqns. (10-11) derive from the fact that the sum of the controls exerted by those enzymes on the RNA species that affect translation equals zero [eqn. (8)]. An important new theorem is an analogue of the expression for the control coefficient of a flux with respect to the concentration of an external modifier (Kacser & Burns, 1979):

$$\Gamma_{mRNA}^{protein} = \varepsilon_{mRNA}^{translation} \cdot C_{translation}^{protein} \tag{12}$$

This theorem is expressed here for the control exerted by the concentration of mRNA on the concentration of a protein. It states that the control coefficient equals the arithmetic product of the elasticity coefficient of translation with respect to the mRNA and the control coefficient of the protein concentration with respect to translation.

The control exerted by transcription on the concentration of a metabolite involves three levels of the hierarchy. In quantitative terms this results in the corresponding control coefficient being equal to the mathematical product of three control coefficients, one for each level of the hierarchy:

$$\Psi_{transcription}^{metabolite} = Y_{transcription}^{mRNA} \cdot \Gamma_{mRNA}^{enzyme} \cdot C_{enzyme}^{metabolite} \tag{13}$$

Capitals Y, Γ and C are used for control coefficients at the levels of RNA, protein and substrate metabolism, respectively. For each of these an expression of the type of eqn. (12) exists. Ψ is used for the control coefficient that runs through the three hierarchies. Eqn. (13) is relatively simple because it was assumed that only the transcription of a single gene was modulated. In the case that the transcription of more than one gene is modulated, complex

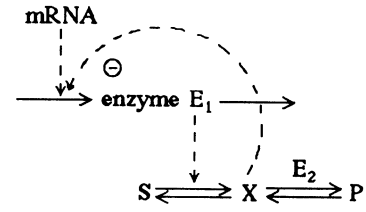


Figure 4. A scheme with feed-back repression. For simplicity, the repression is assumed to operate at the level of translation.

products may arise. The control by an external modifier of transcription is found by multiplying $\Psi^{\text{metabolite}}_{\text{transcription}}$ by the elasticity coefficient of transcription for that modifier:

$$\Psi^{\text{metabolite}}_{\text{modifier}} = \Psi^{\text{metabolite}}_{\text{transcription}} \cdot \varepsilon^{\text{transcription}}_{\text{modifier}} \tag{14}$$

Variable Gene Expression: a Case of Democratic Hierarchy of Control

Up to this point, we assumed that the hierarchical control structure was "dictatorial", i.e. that higher levels of control were insensitive to changes at the lower levels of control. We shall now consider an example of "democratic" control, where the concentration of a metabolite X feed-back represses the synthesis of the enzyme that produces it (Fig. 4). For simplicity we shall assume that the feedback repression occurs at the level of translation and that the concentration of the mRNA is constant. We consider the effect of a change $\mathrm{d}\ln k_{\text{translation}}$ in the inherent rate constant of translation (for instance due to a change in ribosomal concentration). The resulting change in [X] consists of two terms. One is the direct effect of the change in inherent translational activity, the other is the subsequent effect of the change in translation activity due to the change in [X]:

$$\begin{aligned} \mathrm{d}\ln[\mathrm{X}] = C^{[\mathrm{X}]}_{\text{translation}} \cdot \mathrm{d}\ln k_{\text{translation}} &= (C^{[\mathrm{X}]}_1 \cdot \Gamma^1_{\text{translation}} + C^{[\mathrm{X}]}_2 \cdot \Gamma^2_{\text{translation}}) \cdot \mathrm{d}\ln k_{\text{translation}} \\ &+ (C^{[\mathrm{X}]}_1 \cdot \Gamma^1_{\text{translation}} + C^{[\mathrm{X}]}_2 \cdot \Gamma^2_{\text{translation}}) \cdot \varepsilon^{\text{translation}}_{\mathrm{X}} \cdot \mathrm{d}\ln[\mathrm{X}] \end{aligned} \tag{15}$$

Here the subscripts 1 and 2 refer to enzymes 1 and 2 in Fig. 4. Consequently, the control exerted by translation on the concentration of the metabolite X $C^{\mathrm{X}}_{k_{\text{translation}}}$ is (usually) decreased due to the feed-back repression by X ($\varepsilon^{\text{translation}}_{\mathrm{X}}$ is usually negative):

$$C'^{\mathrm{X}}_{k_{\text{translation}}} = \frac{C^{\mathrm{X}}_{\text{translation}}}{1 - \varepsilon^{\text{translation}}_{\mathrm{X}} \cdot C^{\mathrm{X}}_{\text{translation}}} = \frac{C^{\mathrm{X}}_{\text{translation}}}{1 - \varepsilon^{\text{translation}}_{\mathrm{X}} \cdot C^{\mathrm{X}}_1 \cdot \Gamma^1_{\text{translation}}} \tag{16}$$

where we have assumed that the feed-back repression by X on translation only affects the synthesis of enzyme 1 and not that of enzyme 2. $C^{\mathrm{X}}_{\text{translation}}$ corresponds to the ratio of the actual change in ln[X] to the *actual* change in translation activity (including the effect of the feed-back repression). $C'^{\mathrm{X}}_{k_{\text{translation}}}$ corresponds to the ratio of the actual change in ln[X] to the *initial* change in translation activity (excluding the effect of the feed-back repression). A potentially more confusing phenomenon is that the control coefficients of the metabolic

enzymes with respect to metabolite concentrations are also affected by the presence of feed-back repression. Denoting the control coefficients in the presence of feed-back repression by a prime ', one finds (Westerhoff, in preparation):

$$C'^{X}_{1} = \frac{C^{X}_{1}}{1 - \varepsilon^{translation}_{X} \cdot C^{X}_{1} \cdot \Gamma^{1}_{translation}} \tag{17}$$

(Again we assume that feed back repression by X is limited to the synthesis of enzyme 1). C^{X}_{1} is the control coefficient of [X] with respect to enzyme 1 in the absence of feed-back repression (it should be noted that [X] remains a function of the concentrations of enzymes 1 and 2). C'^{X}_{1} is the actual change in ln[X] divided by the change in logarithm of the *initial* change in enzyme 1 concentration, $d\ln[E_1]_0$; in the case of C^{X}_{1} (no prime!) it is divided by the ultimate change in enzyme 1 concentration, $d\ln[E_1]_\infty$:

$$d\ln[X] = C'^{X}_{1} \cdot d\ln[E_1]_\infty = C'^{X}_{1} \cdot d\ln[E_1]_0 \tag{18}$$

The two changes in enzyme concentration are related by:

$$d\ln[E_1]_\infty = d\ln[E_1]_0 + \Gamma^{1}_{translation} \cdot \varepsilon^{translation}_{X} \cdot d\ln[X] \tag{19}$$

Since $\varepsilon^{translation}_{X}$ is usually negative (feed-back *repression*), eqn. (17) reflects the fact that the control exerted by enzyme 1 on the concentration of metabolite X is *reduced* by the presence of the feed-back repression.

Perhaps surprisingly, the absence of feed-back repression (or induction) of X on the synthesis of enzyme 2 ($\Gamma^{2}_{translation} = 0$ in our example) does not imply that the control coefficient with respect to enzyme 2 is the same as in the absence of feed-back repression:

$$C'^{X}_{2} = \frac{C^{X}_{2}}{1 - \varepsilon^{translation}_{X} \cdot C^{X}_{1} \cdot \Gamma^{1}_{translation}} \tag{20}$$

The classical summation theorem holds for either type of control coefficients:

$$C^{X}_{1} + C^{X}_{2} = 0 = C'^{X}_{1} + C'^{X}_{2} \tag{21}$$

Using the method of Westerhoff and Chen (1984), we derived the following connectivity theorem for the primed control coefficients (Westerhoff, in preparation):

$$C'^{X}_{1} \cdot \varepsilon^{1}_{X} + C'^{X}_{2} \cdot \varepsilon^{2}_{X} + \Gamma^{1}_{translation} \cdot C'^{X}_{1} \cdot \varepsilon^{translation}_{X} = -1 \tag{22}$$

By inserting eqns. (17) and (20), it can be shown that this connectivity theorem is consistent with the purely metabolic connectivity theorem (Westerhoff & Chen, 1984), which remains valid:

$$C^{X}_{1} \cdot \varepsilon^{1}_{X} + C^{X}_{2} \cdot \varepsilon^{2}_{X} = -1 \,. \tag{23}$$

Histone Gene Expression in Early Development

In metabolic control theory, systems are assumed to be at steady state. That is, fluxes and concentrations are constant in time. For systems at constant volume, the latter implies that amounts are also constant, i.e. that net synthesis equals net degradation. In growing systems, the latter equality does not apply. However, metabolic control theory has been shown to apply to steadily growing systems, provided that the so-called fluxes to expansion are taken into consideration, i.e. the rates of net synthesis necessary to supply all progeny cells with the normal level of metabolites (Kacser, 1983). In many actual cases, the flux to expansion is a minor component to metabolism. We have studied a case where this flux to expansion is a major component and where its (large) magnitude is essential for the survival of the organism. This is the case of histone gene expression in the early development of some eukaryotic organisms. Histones are proteins needed in large amounts (1:1 mass:mass relative to DNA) to structure eukaryotic DNA. The cell cycle of some eukaryotes in early development is almost as short as that of rapidly growing *E. coli,* yet their cellular DNA content is a thousand times higher. This implies an enormous rate of DNA synthesis and, since all DNA seems to be packaged around histone octamers, an equally impressive requirement of histone protein.

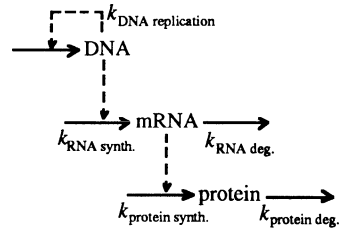


Figure 5. Scheme for histone gene expression. It will be assumed that the rate constants are independent of time, that transcription and DNA synthesis rates are proportional to the amount of DNA, translation and RNA degradation rates proportional to the amount of RNA and proteolysis proportional to the amount of protein. Magnitudes of the rate constants and of the initial amounts of histone protein and histone mRNA were given in Koster *et al.* (1988).

In *Xenopus laevis,* fertilization is followed by a short lag, but then exponential growth (in the sense of DNA replication) sets in, with the constant cell-cycle time of 35 min (Newport & Kirschner, 1982). For such exponential growth, the differential equations corresponding to Fig. 5 can be solved; if one assumes time-independent rate constants. By dividing the amount of protein by the amount of DNA, neglecting the amounts of RNA and protein present in the beginning, one finds for the amount of protein per cell (Koster, 1987):

$$\frac{[\text{protein}]}{[\text{DNA}]} = \frac{n_{\text{genes}} \cdot k_{\text{protein synthesis}} \cdot k_{\text{RNA synthesis}}}{(k_{\text{DNA replication}} + k_{\text{RNA degradation}})\,(k_{\text{DNA replication}} + k_{\text{protein degradation}})} \qquad (24)$$

Here n_{genes} is the number of genes per haploid genome encoding the protein under study, and the rate constants k refer to the processes indicated.

Differentiating the logarithm of this expression with respect to that of $k_{\text{RNA synthesis}}$ one finds the value of 1.0 for the control coefficient of the protein/DNA ratio with respect to RNA synthesis. Similarly, the control coefficients with respect to protein synthesis and the

number of genes per genome both equal 1. For the control coefficients with respect to protein and RNA degradation one finds $-k_{\text{protein degradation}}/(k_{\text{DNA replication}} + k_{\text{protein degradation}})$ and $-k_{\text{RNA degradation}}/(k_{\text{DNA replication}} + k_{\text{RNA degradation}})$ respectively, whereas the control coefficient with respect to DNA replication amounts to

$$-\frac{k_{\text{DNA replication}}}{k_{\text{DNA replication}} + k_{\text{protein degradation}}} - \frac{k_{\text{DNA replication}}}{k_{\text{DNA replication}} + k_{\text{RNA degradation}}}$$

Summing all the control coefficients for rate constants (processes), one finds that the summation theorem applies: the sum of the controls over the amount of protein per cell (i.e., per amount of DNA) adds up to zero. As discussed above, when taken by hierarchical level, the summation theorems also hold, provided that the effect of DNA synthesis is taken into consideration. For instance, the sum of the control coefficients by transcription, RNA degradation, and DNA replication insofar as RNA synthesis is concerned [which gives a control coefficient for DNA replication of $-k_{\text{DNA replication}}/(k_{\text{DNA replication}} + k_{\text{RNA degradation}})$] equals zero.

Calculation of the histone-protein to DNA ratio from the above equation for the case of early development of *Xenopus laevis* led to the ratio of 0.19 (Koster, 1987; cf. Koster *et al.*, 1988). Clearly, exponential growth plus *de novo* synthesis of histone mRNA and histone protein would lead to shortage of histone proteins. As detailed elsewhere (Koster *et al.*, 1988; cf. Woodland, 1980) *Xenopus laevis* deals with this problem by storing histone mRNA and histone protein in its oocyte and by limiting the period of exponential growth to the first 8 hours after fertilization.

Control of and by DNA Supercoiling and the Cellular Energy State

In prokaryotes, gene expression is affected by DNA supercoiling (Drlica, 1984). DNA supercoiling is increased (made more negative) by DNA gyrase, at the cost of the hydrolysis of intracellular ATP. Topoisomerase I decreases (negative) supercoiling. This system is homeostatically controlled both at the level of the degree of supercoiling affecting the activities of gyrase and topoisomerase I and at the level of supercoiling affecting the expression of the gyrase and the topoisomerase I genes (Menzel & Gellert, 1983; Tse-Dinh, 1985). On top of this, there are environmental influences on DNA supercoiling, the mechanism of which has not been elucidated (Higgins *et al.*, 1988).

If one neglected the influences of supercoiling on the expression of the gyrase and topoisomerase I genes, the following summation and connectivity theorems would be valid:

$$C^{\text{supercoiling}}_{\text{gyrase}} + C^{\text{supercoiling}}_{\text{topoisomerase I}} = 0 \tag{25}$$

$$C^{\text{supercoiling}}_{\text{DNA gyrase}} \cdot \varepsilon^{\text{DNA gyrase}}_{\text{supercoiling}} + C^{\text{supercoiling}}_{\text{topoisomerase I}} \cdot \varepsilon^{\text{topoisomerase I}}_{\text{supercoiling}} = -1 \tag{26}$$

The degree of supercoiling may be defined in terms of the change in linking number relative to the relaxed state of the DNA.

In reality the expression of gyrase and topoisomerase I genes are affected by DNA supercoiling. We indicate this by writing the activity g of gyrase as a function of the endogenous activity g_0 and the degree of supercoiling σ:

$$g = g(\sigma, g_0) \tag{27}$$

Similarly we write for the activity I of topoisomerase I:

$$I = I(\sigma, I_0) \tag{28}$$

The degree of supercoiling remains the usual function of the overall activities of gyrase and topoisomerase I:

$$\sigma = \sigma(g, I) \tag{29}$$

Note that we leave out of consideration the influences of transcription on supercoiling, which have recently been shown to be important (Wu *et al.*, 1988; Figueroa & Bossi, 1988). The usual control coefficient indicating the control of gyrase on supercoiling is:

$$C_g^\sigma \equiv \left(\frac{\mathrm{d}\ln\sigma}{\mathrm{d}\ln g}\right)_I \tag{30}$$

That is, this control coefficient relates the change in supercoiling to the change in gyrase activity, the latter including the secondary change in gyrase activity resulting from the effect of supercoiling on gyrase expression. C_g^σ differs from the effect on DNA supercoiling taken per amount of initial change in gyrase activity, as defined by

$$C'^{\sigma}_{g} \equiv \left(\frac{\mathrm{d}\ln\sigma}{\mathrm{d}\ln g_0}\right)_{I_0} \tag{31}$$

where it is noted that there should be no *initial* change in topoisomerase I activity, but a change due to the change in supercoiling is allowed. For the change in gyrase concentration we may write:

$$\mathrm{d}\ln g = \mathrm{d}\ln g_0 + Y^g_\sigma \mathrm{d}\ln\sigma \tag{32}$$

Here Y^g_σ is the control coefficient for the effect of a change in supercoiling on the concentration of DNA gyrase. With similar definitions relating to topoisomerase I, one can express the two control coefficients for gyrase on supercoiling into one another:

$$C'^{\sigma}_{g} = \frac{C_g^\sigma}{1 - C_g^\sigma \cdot Y^g_\sigma - C_I^\sigma \cdot Y^I_\sigma} \tag{33}$$

The summation and an adjusted connectivity theorem are valid for the new control coefficients (Westerhoff, in preparation). These can be used to express the new control coefficients

for the effect of gyrase on supercoiling into more fundamental properties:

$$1/C'^{\sigma}_{g} = -\varepsilon^{g}_{\sigma} + \varepsilon^{l}_{\sigma} - Y^{g}_{\sigma} + Y^{l}_{\sigma} \tag{34}$$

Not unexpectedly, feed-back repression (negative Y^{g}_{σ}) has an effect similar to that of feed-back inhibition (negative ε^{g}_{σ}); it reduces the control of supercoiling by DNA gyrase (it increases C'^{σ}_{g}). DNA gyrase can decrease the linking number of negatively supercoiled, double-stranded closed circular DNA. The free-energy source for this is the ATP that is hydrolysed by the enzyme. In vitro, supercoiling of a plasmid by DNA gyrase, was a rather strong function of the Gibbs energy of hydrolysis of ATP (Westerhoff *et al.*, 1988). To obtain an indication if the same phenomenon may occur *in vivo,* we incubated *E. coli* in the presence of a protonophore, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), which is known to decrease the intracellular free energy of hydrolysis of ATP. Depending on the amount of protonophore added, we found an increase in average linking number (corresponding to a decrease in negative supercoiling) of the intracellular reporter plasmid pBR322 of up to 9. In terms of control theory, such a control by intracellular ATP is written as follows:

$$C'^{\sigma}_{ATP} = \frac{\varepsilon^{g}_{ATP}}{-\varepsilon^{g}_{\sigma} + \varepsilon^{l}_{\sigma} - Y^{g}_{\sigma} + Y^{l}_{\sigma}} \tag{35}$$

Eqn. (35) demonstrates that this control is quite a complex function of various enzyme properties as well as of the properties of the expression of the gyrase genes. This is found even though we have neglected the effects of transcription on supercoiling and even though we have assumed that ATP only affects supercoiling through DNA gyrase. Actual transcription is not essential for the effect of the protonophore on DNA supercoiling (Westerhoff, unpublished observation).

Regulation versus Control

Confusion has arisen with regard to what metabolic control analysis does and does not discuss. In its generic form, metabolic control analysis discusses the effects of changes in system parameters on system variables. Most often the variables have been fluxes and concentrations, the parameters have been enzyme concentrations. Limiting the present discussion to fluxes, metabolic control analysis allows one to determine and explain the extent to which potential changes in enzyme activities or in the concentration of externally determined concentrations of metabolic modifiers, affect a flux.

It is important to note that metabolic control analysis does not therefore analyse (i) how the flux is actually regulated, or (ii) which regulatory links in metabolism are important for the actual regulation of the flux. The former question (i) cannot be addressed without knowing what actually changes in the external factors that affect metabolism (i.e, which of the enzymes are and which are not activated in the *in vivo* situation).

The second question (ii) can be addressed to some extent by the following extension of metabolic control theory. We consider the example of Fig. 4 and eqn. (16). Here we found the control in the absence of changes in gene expression to be given by $C^{X}_{translation}$ and the control for the case where the derepression of gene expression is operative as $C'^{X}_{k_{translation}}$. Clearly, in eqn. (16) the regulatory importance of the dashed regulatory loop in Fig. 4 that leads from X to transcription and then back to X is given by what we here define as the regulation coefficient R for that loop:

$$R = C^{X}_{1} \cdot \Gamma^{1}_{translation} \cdot \varepsilon^{translation}_{X} \tag{36}$$

Other examples of coefficients for regulatory loops are found in the denominator of eqn. (33).

One can also quantify values for regulatory *links*. For instance the extent to which the activity of translation regulates the concentration of X (note that we can discuss in terms of regulation by translation rather than control by translation because the activity of translation now is variable due to the feed-back repression by X) is quantified by

$$R^{X}_{translation} = C^{X}_{1} \cdot C^{1}_{translation} \tag{37}$$

To demonstrate that these quantifications of regulatory links can be used to actually calculate control coefficients, it serves to rewrite eqn. (16) as follows:

$$C'^{X}_{k_{translation}} = C^{X}_{translation} + C'^{X}_{k_{translation}} \cdot \varepsilon^{translation}_{X} \cdot C^{X}_{translation} \tag{38}$$

Inspection of Fig. 4 confirms that there are two regulatory links leading from translation to X. The first is the straightforward connection through enzyme 1, stopping in X. This yields the regulatory coefficient $C^{X}_{translation}$ (which is equal to the control coefficient in the absence of feedback repression). The second link involves the feed back repression: it runs from translation through enzyme 1 to X (giving rise to $C^{X}_{translation}$) then back from X to translation (giving rise to $\varepsilon^{translation}_{X}$) and then again from translation to X. Because this second link again consists of a direct effect and a secondary effect involving feed back repression, it invokes the factor $C'^{X}_{k_{translation}}$ in addition to $C^{X}_{translation}$.

Discussion

In the above, we have expanded control theory in a first attempt to include variable gene expression. It turns out that control coefficients that include variations in gene expression differ from control coefficients that do not include them. When comparing control coefficients determined by varying gene dosage to control coefficients determined using metabolic inhibitors, this should be kept in mind. Biochemical systems theory has also dealt with variations in gene expression (Savageau, 1976), mostly in the integral sense. In our experience however, the parameter choice in metabolic control theory is more amenable to

experimental analysis than the one in biochemical systems theory (for a more detailed comparison of the application of various control theoretic methods see Chapter 6 by Groen and Westerhoff in this book). Consequently, we consider it useful to extend metabolic control theory into the domain of varying gene expression. Such an extension may allow improved interpretation of control data already available (e.g. Barthelmess *et al.*, 1974) and stimulate further experimental control analyses.

In some organisms, histone gene expression in early development is a race against the clock. If growth continued to be exponential, the embryo would fail to provide sufficient histones to package its newly synthesized DNA. However, as detailed elsewhere (Koster *et al.*, 1988), growth slows down in time.

The control of intracellular supercoiling by the cellular energy state is likely to be complicated. It may involve alterations in transcription rates (Wu *et al.*, 1988), alterations in gyrase activity and subsequent alteration in supercoiling. The latter may again affect the expression of the gyrase genes, resulting in a tendency towards homeostasis (Menzel & Gellert, 1983).

Workers expecting that metabolic control theory is devised to solve the alpha and omega of metabolic regulation may have been disappointed. Indeed, metabolic control theory is limited to discussing what effect changes of certain properties of metabolic systems may have on fluxes and concentrations. The question of which changes actually occur in a certain physiological context cannot be solved by a general theory, but must involve detailed knowledge of the system under study.

However, metabolic control theory can do more than what has been focussed on traditionally. We have shown here that metabolic control theory can quantify the importance of regulatory links within metabolic pathways. If more than one such link exists, one can use our method to decide which is the most important. This may lead to increased understanding of why metabolic pathways that are controlled have certain control structures (Savageau, 1976; Westerhoff & Van Dam, 1987).

REFERENCES

Barthelmess, I. B., Curtis, C. F. & Kacser, H. (1974) *J. Mol. Biol. 87,* 303-316
Dean, A. M., Dykhuizen, D. E. & Hartl, D. L. (1986) *Genet. Res. Camb. 48,* 1-8
Drlica, K. (1984) *Microbiol. Rev. 48,* 273-289
Figueroa, N. & Bossi, L. (1988) *Proc. Natl. Acad. Sci. USA 85,* 9416-9429
Flint, H. J., Tateson, R. W., Barthelmess, I. B., Porteous, D. J., Donachie, W. D. & Kacser, H. (1981) *Biochem. J. 200,* 231-246
Heinrich, R., Rapoport, S. M. & Rapoport, T. A. (1977) *Prog. Biophys. Mol. Biol. 33,* 99-187
Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G. & Bremer, E. (1988) *Cell 52,* 569-584
Kacser, H. (1983) *Biochem. Soc. Trans. 11,* 35-40
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Kacser, H. & Burns, J. A. (1979) *Biochem. Soc. Trans. 7,* 1149-1160
Kacser, H. & Porteous, J. W. (1987) *Trends Biochem. Sci. 12,* 6-14
Kell, D. B. & Westerhoff, H. V. (1986) *FEMS Microbiol. Rev. 39,* 305-320
Koster, J. G. (1987) Ph. D. Thesis, University of Amsterdam

Koster, J. G., Destrée, O. H. J. & Westerhoff, H. V. (1988) *J. theor. Biol. 135,* 139-167
Menzel, R. & Gellert, M. (1983) *Cell 34,* 105-113
Newport, J. & Kirschner, M. (1982) *Cell 30,* 675-686
Savageau, M. A. (1976) *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*, Addison-Wesley, Reading, Massachusetts
Tse-Dinh, Y.-C. (1985) *Nucl. Acids Res. 13,* 4751-4763
Walsh, K. & Koshland, D. E., Jr. (1984) *Proc. Natl. Acad. Sci. USA 82,* 3577-3581
Westerhoff, H. V. (1989*a*) *Biochimie 71,* 877-886
Westerhoff, H. V. (ed.) (1989*b*) *Principles of Metabolic Control,* Plenum Press, New York
Westerhoff, H. V. & Chen, Y. (1984) *Eur. J. Biochem. 142,* 425-430
Westerhoff, H. V. & Van Dam, K. (1987) *Thermodynamics and Control of Biological Free-Energy Transduction,* Elsevier, Amsterdam
Westerhoff, H. V., O'Dea, M. H., Maxwell, A. & Gellert, M. (1988) *Cell Biophys. 12,* 157-181
Woodland, H. (1980) *FEBS Lett. 121,* 1-17
Wu, H.-Y., Shyy, S., Wang, J. & Liu, L.F. (1988) *Cell 53,* 433-440

Chapter 36

An Experimentalist's View of Control Analysis

DANIEL E. ATKINSON

IN RESPONDING to the invitation to comment on some aspects of the symposium, I will attempt no evaluation of the internal logic or mathematical merits of the various mathematical treatments that have been discussed. Those and other characteristics of these treatments have been discussed by other participants. Rather, I will focus my comments mainly on two questions that Dr. Cornish-Bowden asked me to consider, and which he posed in his introductory presentation (see Chapter 1): (a) what characteristics should a theory have if it is to be useful to experimentalists and (b) are the main treatments that have been discussed here — those of Kacser and Burns (see especially Chapters 3, 17, 20 and 25), Heinrich and Rapoport (Chapter 28), and Savageau (Chapters 4 and 5) — valuable to experimental metabolic biochemists?

We all believe in theory, and I hope that we all believe in experiment. Some participants in the symposium might add a caveat; they believe in experiments as long as they are not expected to perform any. Probably we all agree that the importance of experiments and their results lies not in themselves, but in their contribution to expanding our understanding, and that the aim of science is greater explanatory power — that is, the construction of generalizations that relate in a more definitive way to a wider range of phenomena.

Several words that are central to this discussion have quite different meanings to different participants in this symposium and to members of the biochemical community more generally. Attempts to suggest answers to the questions posed by Dr. Cornish-Bowden are likely to founder in semantic confusion unless we begin with a consideration of those differences. Specifically, the words "theory" and "control" are used by various participants with very different meanings.

A major aim of science is not only to learn more about the world, but to understand more about it. Understanding primarily means integration and interrelation of as wide a range of phenomena as possible in terms of explanatory generalizations. These generalizations are given many names, such as hunches, working hypotheses, hypotheses, theories, and

Daniel E. Atkinson ● Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024, USA

Control of Metabolic Processes
Edited by A. Cornish-Bowden and M. L. Cárdenas
Plenum Press, New York, 1990

laws. I will use the generic term "generalizations" except where "theory" is mandated by Dr. Cornish-Bowden's charge (Chapter 1) or the usage of other participants.

In order to correlate, explain, or predict phenomena, a scientific generalization must of course deal with the subject matter of the field to which it applies. Genetic theories or generalizations deal with and interrelate genetic observations, physical theories deal with physics, geological theories with geology, and so on. The fact that the generalizations of a field deal with the subject matter of the field is so obvious that it would ordinarily seem superfluous to mention it. But it is a remarkable fact that the treatments of Kacser and Burns and of Heinrich and Rapoport have no specifically metabolic or biochemical content. They deal mathematically with generalized sequences of a few reactions, which could equally well be organic or inorganic, biological or geological. They are systems for mathematical treatment of kinetic results, and their constraints are those of the mathematical formalisms that are employed, rather than those of biological systems — or for that matter, of any other specific kind of systems. Having no specific relation to any kind of phenomena or observation, these treatments not only are not biochemical, they are not really scientific theories or generalizations at all. As Dr. Kacser remarked at this symposium, they are methods of data treatment. Use of the term "theory" in this context is unfortunate.

Abstraction and remoteness from the world of real observations is a deliberate feature of the design of these systems. Those who work with them speak of the desirability of designing a system without preconceptions; that is, of first setting up a completely general treatment and only at the time of use making specific adjustments if necessary. Nothing could be farther from the way that ordinary scientific generalizations are developed. Generalizations deal with observations, and make specific statements about observations and their relationships. To the best of my knowledge, no one has ever set out to design a geological theory devoid of geological content or preconceptions, or a chemical theory without chemical input.

We can conclude at this stage that these treatments, having no discipline-specific content or predictive power, cannot aid an experimentalist in posing questions or devising experiments to try to answer them, and thus in contributing to our understanding of metabolic regulation. They are also not relevant to interpretation of his results. That conclusion is related to the questions that Dr. Cornish-Bowden posed for me.

It is said that these treatments introduce a quantitative approach into an area — the study of metabolic regulation — that has hitherto been qualitative. The unspoken implication is that quantitative is better than qualitative. That assumption is not always valid. Qualitative questions are questions of kind; quantitative questions are questions of degree. It is generally useful to know what we are dealing with before we attempt to measure it. If a man walking along a path hears a rustle in the tall grass and catches a glimpse of tawny skin, the qualitative question of whether the creature is a tiger or an antelope is of more immediate importance to him than a precise quantitative measurement of its body length or height at the shoulders. Similarly, in almost any context, qualitative questions are more fundamental than quantitative questions, and logically must have priority. Even when methods are quantitative or semi-quantitative, the important questions to be addressed are qualitative. A metabolic chemist measures enzyme properties or the distribution of radioactivity among metabolites not for the sake of quantitation *per se*, but in an attempt to answer the underlying

qualitative questions. What reactions are involved? How are they related in the cell? Into what sequences are they organized? How do the sequences interact? How are they regulated? In developing answers to these and similar questions, quantitation is a means to an end — understanding — rather than a meaningful goal in itself. And a system that is quantitative but lacks specific substantive content can only be, as these systems are, methods for dealing with numbers rather than scientific generalizations or theories.

The fact that these treatments have no biochemical or predictive content does not necessarily mean that they might not be of value to experimentalists. Statistical techniques are similarly devoid of discipline-specific content, but are useful to experimentalists in nearly all fields. The mathematical treatments discussed at this symposium have more in common with statistical techniques than with scientific theories or generalizations. Thus we must ask whether, like statistics, they can aid an experimentalist in designing experiments (not in terms of what questions to ask — that is the province of scientific generalizations — but of how to ask them efficiently) and in evaluating the probable significance of experimental results. The remainder of this chapter will deal in one way or another with that question.

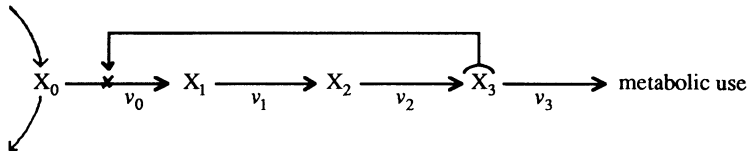
The study of metabolism has led to such generalizations as: regulation is usually exerted at the first committed step of a sequence, which is the same as saying that it occurs at branch-points; the enzyme property that is usually regulated is the affinity of the branch-point enzyme for a substrate for which it competes with the enzyme that catalyses the first reaction of the other branch; the reactions catalysed by such regulated enzymes are usually of high kinetic order with respect to substrate; responses to modifier metabolites are also usually of high order; and so on. Discovery of none of those features could have been facilitated by use of the treatments with which this symposium dealt, and none of them are incorporated into those treatments. Indeed the treatments appear to be unable to deal with some of those features of real systems.

The treatment of Savageau (Chapters 4 and 5) begins with a consideration of enzymes, and uses power functions to approximate some features of their behaviour. All subsequent manipulations are in terms of derived mathematical terms. It thus at the outset divorces itself, for the sake of mathematical convenience, from the actual physical properties and functional parameters of enzymes and metabolic systems. Since those are the features of the system about which an experimentalist must think when attempting to understand metabolic function, correlation, integration, and regulation, this approach interposes itself between observations and meaningful consideration of their biological significance and surely, if used by an experimentalist, would impede his generation of explanatory hypotheses.

In discussions of this method, the rates of all reactions that diverge from a branch-point are lumped together for the sake of mathematical convenience. Nearly all known metabolic regulation is effected by altering the partition ratio at branch-points — that is, by changing the relative rates of the reactions that diverge from the branch-point. A method that lumps those reactions for the sake of elegance or convenience in mathematical analysis thus appears to lose in the process any possibility of dealing with actual metabolic regulation. Such lumping is not of course a necessary feature of the method, but its use by those who promote the method seems to indicate indifference toward actual regulatory patterns in real cells.

In this discussion I will deal specifically with the treatment of Kacser and Burns and assume that my comments apply in general also to that of Heinrich and Rapoport, since those treatments are considered by both groups to be virtually interchangeable.

These treatments deal with a formalized sequence of a few reactions, which may include a feedback loop, as in Scheme 1, where a later metabolite is a negative modifier for the enzyme that catalyses the conversion of X_0 to X_1:



Scheme 1

This treatment defines for each enzyme a flux control coefficient, essentially the partial derivative $\partial \ln F/\partial \ln a_i$ where F is flux through the system at steady state and a_i is the activity of the enzyme that catalyses reaction i. In the absence of a feedback loop and if only small variations are considered, it may reasonably be assumed that such expressions are true derivatives, that is, that they may be integrated and have other properties of such functions. Among the consequences of that assumption is that the flux control coefficients for the enzymes of a sequence must sum to a value of one. That feature — summation to one — is a central postulate of the treatment. It is a mathematical property of functions of the kind that are employed in this treatment, not a biological generalization. It seems to be tacitly assumed in most discussions of these treatments that the flux control coefficient of an enzyme is, as the name implies, a measure of the extent to which that enzyme influences the flux through the sequence. As I noted in the Prologue, that assumption cannot be true when the enzyme is of high order and is regulated by change of its affinity for substrate. Indeed the very properties that allow an enzyme to be a sensitively-responding controller of flux — high kinetic order and variable affinity for substrate — cause it to have a low value of the flux control coefficient as that parameter is defined, as pointed out by Sauro & Fell (1987) and by me in the Prologue.

When we consider a system containing a feedback loop, it becomes impossible to separate variables, since the concentration of the end-product feedback modifier X_3 is a function of flux, and flux is a function of the concentration of the feedback modifier. The treatment thus appears not to be applicable to feedback-controlled systems, which include nearly all biochemical sequences. As we will see later, in a properly-functioning feedback-regulated system, whether technological or biological, the value of all flux control coefficients are zero, since small changes in the concentration of any enzyme will not affect the regulated flux. This is true regardless of the order of reaction and of what property of the regulatory enzyme is modulated. These treatments explicitly ignore or deny that fact. They also lump the net demand, symbolized in Scheme 1 by the arrow showing metabolic use of X_3, with the other reactions of the sequence. Such an approach confounds the regulatory interactions by lumping the control system with the factors to which it responds.

Failure to distinguish between the regulatory properties of a sequence and the external factors to which the regulatory system responds is the consequence of an approach that concerns itself indiscriminately with all factors that affect the flux through a sequence without regard for their nature or functional significance. We encounter here a fundamental difference in approach between those who work with the mathematical treatments under discussion and those who attempt to understand the regulatory interactions of actual metabolic systems. On the basis of this symposium, I have reluctantly become convinced that the difference is irreconcilable.

Those whom Dr. Cornish-Bowden terms theoreticians (although the mathematical treatments with which they work are not theories in the usual scientific sense of the term, as we have noted) are interested equally in all factors that affect the flux. They explicitly do not distinguish between intrinsic properties and extrinsic factors. Thus, despite their use of the word "control," they deal really with anything that *affects* rates. Because they ignore the distinction between external factors that tend to perturb the system and the evolved intrinsic homeostatic properties of the system that resist those perturbations, they are unable to deal with the regulation of real sequences. Metabolic chemists would generally consider the word "control" to refer to factors that are involved in the *regulation* of a flux, not to everything that might *affect* it. They more often use the word "regulate," which avoids ambiguity of that kind. If we are to understand regulation, we must study the regulatory system itself and its reaction to the environment. Reaction to the environment is the essence of regulation or homeostasis, and we handicap ourselves fatally if we begin by lumping the regulatory system and the environment (in this case, the demand for the end product) indiscriminately together.

The situation may be illustrated by consideration of a familiar example of technological regulation, a household refrigerator. At steady state, the rate at which heat is pumped out of the refrigerator is equal to the sum of all of the flows of heat into it. The rate at which heat enters will be determined by many factors, among them the ambient temperature, the efficiency of the insulation of the walls, the tightness of fit of the door, how often the door is opened and for how long, and the temperatures and heat capacities of the materials that are put into the refrigerator. The rate at which heat is pumped out depends primarily on the fraction of time that the compressor runs, and that is the variable that is regulated.

The regulatory system of the refrigerator does not monitor the rate of every heat leak and the amount of heat introduced with everything that is put into it, sum them, and adjust the run time of the compressor so as to exactly balance those heat gains. It accomplishes the goal of balancing heat flows out and in by a much simpler means — it measures the internal temperature and controls the compressor so as to maintain a nearly constant temperature.

Each component of the refrigeration system could become rate-limiting if it were defective. However, in a properly operating system, with heat-pumping capacity greater than the demand on the system, the size of the motor or the compressor, the tensile strength of the drive belt, the area of the radiator, and other features of the system could be changed with no effect on the maintenance of temperature. That is, every component of the system has a capacity greater than is being used, and although all are essential to the regulation of temperature, the flux control coefficient of each of them, and of all combined, is zero.

No one would seriously propose that, in considering the operation of a refrigerator, we

should indiscriminately lump together the heat leak through its walls and the fraction of time that the compressor runs. To do so would be to ignore the design features that make constancy of temperature possible. The factors that affect the entry of heat into the refrigerator are not part of the control system; they are what the system works against. The run cycle of the compressor depends primarily on the rate at which heat leaks in. In the case of a refrigerator, everyone as a matter of course distinguishes between, on the one hand, the factors that tend to change the internal temperature of the refrigerator and, on the other, the operation of the regulatory system that responds to those factors and holds the temperature within a narrow range. That distinction is essential to understanding regulation.

The analogy with a regulated biosynthetic sequence is quite close. The demand for the product of the sequence typically has several components. If the product is an amino acid, for example, it will be used in protein synthesis and perhaps in synthesis of other amino acids or other types of metabolites. It will be generated by degradation of cellular proteins, and may be gained from or lost to the environment (the interstitial fluid and blood in the case of mammalian cells). Like the refrigerator, the regulatory system does not monitor all of these gains and losses of the product, but only its concentration. The properties of a regulatory enzyme, typically the branch-point enzyme that initiates the sequence, are adjusted so as to maintain the concentration of the end product within narrow limits, and thus automatically to balance the rate of production of the product with its momentary demand.

In a typical metabolic sequence, the rates of individual reactions and the flux through the sequence are not determined by the raw properties of the enzymes, but primarily by modulation of the properties of the enzyme that catalyses the first reaction. All enzymes are operating below capacity. The flux will increase or decrease with increased or decreased demand, as reflected in the concentration of the end product (which typically is a negative modifier for the first enzyme), but is not sensitive to changes in the amounts of the component enzymes, singly or together. The flux control coefficient for each enzyme, and thus the sum of the flux control coefficients, is zero. Of course that situation has limits; if the concentration of an enzyme were to be reduced until its maximal velocity was less than the preexisting flux, that enzyme would begin to limit the flux through the system, and its flux control coefficient would rise toward a value of one. It is noteworthy that it is only in such a case, where, in sharp contrast to a normally operating sequence, the amount of an enzyme is a flux-limiting factor, that there is any relationship between the flux control coefficient as defined in these systems and the regulation, control, or limitation of flux. And in such cases regulation would fail.

It is evident that flux control coefficients close to zero are essential if the flux through a sequence is to be regulated in response to metabolic need. As in any feedback-regulated system, flux must reflect demand, not the properties of the component enzymes. Because of the inapplicability of the mathematical treatments under discussion to feedback control, those who propose or use them frequently assume that the concentration of the end product is somehow fixed or clamped. When that assumption is made we no longer deal with metabolic regulation — which necessarily determines both fluxes and concentrations in the absence of a helpful supernatural finger fixing either — and we can only engage in a mathematical exercise.

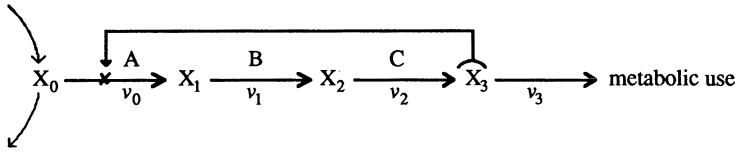
It was suggested at the symposium that even if flux control coefficients for reactions within the feedback loop are all zero, the net demand (the sum of processes that use metabolite X_3, labelled v_3 in Scheme 1), exerts total control and has a flux control coefficient of one. The two components of that suggestion — total control and a control coefficient of one — must be considered separately.

It is valid, in one sense, to say that v_3 exerts total control over flux within the feedback loop, since that flux will be regulated so as to be equal to v_3. That adjustment occurs, however, only as a consequence of modulation of v_0, the rate of the reaction catalysed by the first enzyme of the sequence. Actual control is entirely at that first step. There is no logical or semantic difficulty, however, in the perception that net demand and the first step both exert total control over the flux; we are merely dealing with different levels in the regulatory system.

The second statement, that the flux control coefficient of the demand step will have a value of one, is not correct. The use of the end product metabolite X_3 is, like other metabolic reactions, subject to regulation. Just as for the reactions within the feedback loop, the V_{max} values of the enzyme or enzymes for which X_3 is a substrate will exceed their maximal metabolic rates, and the actual rates of the reactions that they catalyse will be determined by their response to appropriate signals. It is the essence of regulation that fluxes respond to regulatory signals; in such cases the V_{max} values of the enzymes are not relevant (i.e. flux control coefficients are zero) as long as they are large enough to handle the required rates.

Numerical Simulation of Feedback Metabolic Regulation

Figs. 1 to 6 show some characteristics of a regulated biosynthetic sequence as simulated in an iterative numerical model. The model is based on the same idealized reaction sequence as that previously discussed (Scheme 2). Being iterative, it can deal with the circular causality inherent in feedback regulation of fluxes, in which the concentration of the end product is both an input to and a consequence of the regulation.



Scheme 2

The end-product, X_3, is a negative feedback modifier of enzyme A, which converts the branch-point metabolite to the first metabolite specific to this sequence, X_1. Enzyme A has four sites each for substrate and modifier. The values of $S_{0.5}$ and $M_{0.5}$ (the concentrations of substrate X_0 and modifier X_3 respectively for half-saturation of enzyme A) are assignable, as are the cooperativity factors (or the increments or decrements of free energy of binding

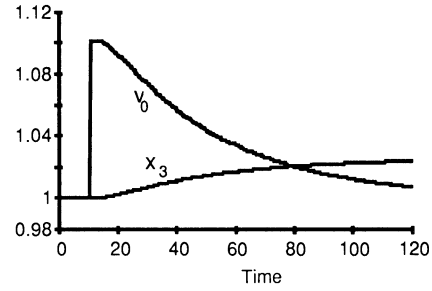


Figure 1. Effect of increasing V_{max} of the first enzyme in the sequence of Scheme 2 by 10%. Values of v_0, the rate of the first reaction, and of $[X_3]$, the concentration of the end product, were obtained by use of the model described in the text. They are plotted in terms of their values in the pre-existing steady state. At ten units on the arbitrary time scale, the maximal velocity of the first enzyme, A, was increased by 10%.

that result from previous binding of another ligand of the same kind at another site) and the interaction factors (or increments or decrements of the free energy of binding) between sites of different kinds — that is, the effect on binding of either substrate (X_0) or modifier (X_3) of previous binding of the other. The reaction catalysed by that enzyme is assumed to be unidirectional. That assumption is reasonable, since it is conceptually necessary that regulatory enzymes of this type catalyse unidirectional reactions (and, of course, because the expectation has been borne out by observation). The net rate at which the product, X_3, is used is assignable, and use of the end-product is assumed to be unidirectional. In the simulations on which these figures are based, the binding of a molecule of substrate or modifier is enhanced by 1.4 kcal/mol by each molecule of the same kind already bound (the cooperativity factor is 10) and binding of a molecule of either type is less favourable by 1 kcal/mol for each molecule of the other that is already bound (a negative binding interaction factor of 5). In each figure the system is initially at steady state and is perturbed by the indicated change at 10 arbitrary time units.

If the activity of the first enzyme is increased by 10% (Fig. 1), there is an instantaneous increase of 10% in the reaction velocity of the reaction catalysed by that enzyme, but the resulting increase in concentration of the regulatory end product adjusts the affinity of the enzyme to bring flux back into exact agreement with the demand for the end-product. At the new steady state the flux is identical with the initial flux and concentrations are slightly altered.

If the activities of all three enzymes are increased simultaneously by 10% (Fig. 2), the pattern is similar, except that the increases in activities of the other enzymes transmit the effect to X_3 a little faster so that the rate of the regulated reaction returns to the steady-state

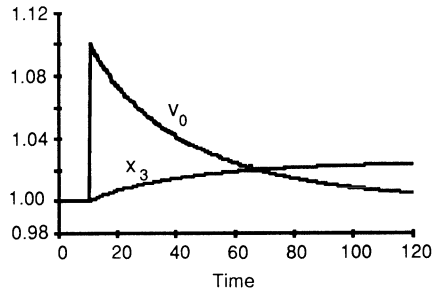


Figure 2. Effect of increasing V_{max} values of all enzymes of the sequence by 10%. Same as Fig. 1, except that the maximal velocities of enzymes A, B, and C were simultaneously increased by 10% at 10 on the time scale.

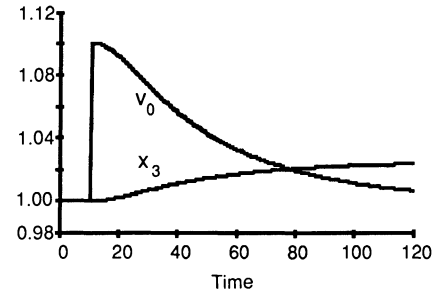


Figure 3. Effect of decreasing the intrinsic value of $S_{0.5}$ for the first enzyme. Same as Fig. 1, except that $S_{0.5}$ was decreased by 2.75% at 10 on the time scale.

flux a bit more quickly. It is evident that the steady-state value of the flux control coefficient is zero for each enzyme, and for all enzymes together.

The response pattern is similar when the rate of the first enzyme is transiently increased by a change in its affinity for substrate, rather than a change in the amount of the enzyme. If the intrinsic value of $S_{0.5}$ (the value of $S_{0.5}$ in the absence of modifier) is decreased so as to cause an increase of 10% in momentary reaction rate (in this simulation, a decrease of 2.75% in $S_{0.5}$ is required) the pattern (Fig. 3) is essentially identical to that of Figs. 1 and 2. A similar result is obtained if the affinity of the enzyme for modifier X_3 is decreased so as to increase the momentary rate by 10% (not shown). Neither of these changes is physiologically relevant; they are included for the sake of completeness and because the flux control coefficient is said to refer also to changes of this kind.

The magnitude of the transient effects of changes in activity of enzymes other than the first will depend on the operating parameters of those enzymes. For the set of parameters that I used, the transient effects on both the rate of the first reaction and the concentration of end product are very small. That will be true for all physiologically reasonable values. In this simulation, if the concentration of the second enzyme, B, is increased by 10%, the concentration of the end product X_3 rises momentarily by about 0.03%, which causes the rate of the first reaction to fall by about 0.13% (Fig. 4). In this case both the flux and the concentration of end product will return exactly to their initial values.

A much more important aspect of regulation is that the flux through a sequence varies in response to need (as indicated by changes in the concentration of the end-product) with relatively little change in the concentration of the end-product. Again the ratio of amounts of change will depend on the parameters of the system and on the concentrations of substrate

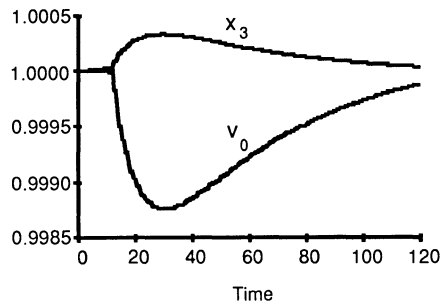


Figure 4. Effect of increasing V_{max} of the second enzyme of the sequence by 10%. Same as Fig. 1, except that the maximal velocity of enzyme B was increased by 10% at 10 on the time scale.

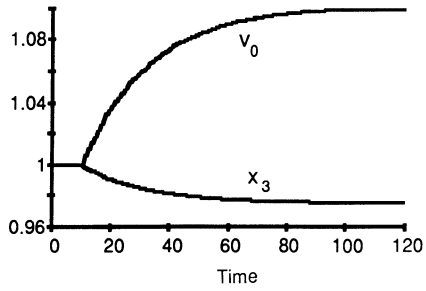


Figure 5. Effect of increasing demand by 10%. The demand (rate of removal of the end product X_3) was increased by 10% at time 10 on the time scale.

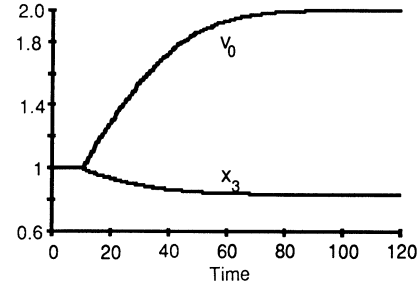


Figure 6. Effect of doubling demand. Same as Fig. 5, except that demand was increased by 100%.

and modifier in comparison with the values of $S_{0.5}$ and $M_{0.5}$. For the parameters I used, which were conservatively chosen to be metabolically reasonable, this kind of control is illustrated by the consequences of increasing demand (the rate of removal of X_3) by 10%. The flux increases by 10% and demand is met, at the expense of a decrease of about 2% in the concentration of the end-product (Fig. 5). The relative pattern is very similar when the demand is doubled (Fig. 6).

Comment on Pre-Symposium Papers

Comment on some of the responses to my pre-symposium paper (reprinted with the responses in the Prologue to this book) seems desirable, since the responses addressed some salient points.

Mazat. Dr. Mazat says that the most important thing that theorists have to say to experimentalists is to urge them to work out for themselves the theory that they need. That is excellent practical advice, but experimentalists do not need such urging. All of the useful generalizations that contribute to our understanding of metabolic regulation have been developed by experimentalists. Like most other scientific generalizations, they have grown directly from observations, and are rich in discipline-specific content. Between 1956 and 1960, many of the basic generalizations on which our understanding of metabolic regulation is still based were proposed by Umbarger (1956) or by Pardee (Yates & Pardee, 1956). Dr. Chock has briefly reviewed at this symposium (see Chapter 13 in this book) the brilliant interplay of experiment and conceptual development that has been so extraordinarily productive in the study of glutamine synthetase by Earl Stadtman's group and others at NIH, and specifically the extensive system of generalizations concerning the properties and functions of regulatory cascades that Chock and Stadtman proposed on the basis of that experimental program. Those generalizations have provided insight into the operation of many other biological systems. Dr. Easterby presented interesting generalizations relating to

transitions between steady states (Chapter 23). Again they were based on experimental findings.

Fell and Sauro. This is not the place for extended discussion of the nature of scientific theories or hypotheses, but the relationship between observation and generalization, introduced into the discussion by Fell and Sauro, is relevant to the matters under discussion. Hypotheses and other generalizations arise from observation, not from divine inspiration. The view of Popper, that scientists are doomed only to attempt to falsify hypotheses that have somehow arisen de novo without relation to observation, seems to me to be as far from the realities of the actual practice of science as it would be possible to get. Of course no one could have guessed in the absence of observation that several di- and tricarboxylic acids are intermediates in the catabolism of sugars, or that all sequences are functionally coupled by the ATP-ADP-AMP system, or that genes exist and are made of nucleic acid, and so on. The catalogue would include the whole of scientific knowledge. Generalizations depend on, and derive from, observations, and the converse is not true.

In general, observations are useful because they lead to the generation of hypotheses, the modification of hypotheses, or the rejection of hypotheses. That is, the goal of science is understanding, not accumulation of facts. Fell and Sauro quote Darwin to that effect. But they misconstrue his meaning when they add that if anyone believes that there is a significant role in science for pure observation uncontaminated by any theory or hypothesis he is disagreeing with Darwin. A more inappropriate opponent of the importance of pure observation could hardly be imagined. On the basis of observations that were completely uncontaminated by any theory or hypothesis, Darwin was led to the most important unifying generalization in biology, and perhaps the most significant contribution to scientific understanding of all time. When he was surprised to find that the fossils of South America resembled living animals on that continent much more closely than they resembled fossils of Europe and Asia (he had expected that organisms living at the same time anywhere on the earth would be similar), and that cave organisms resembled organisms of the vicinity more than cave organisms on other continents, he was not gathering evidence for or against any hypothesis. Although he implicitly accepted the individual creation of species, it most certainly never occurred to him that he was gathering evidence to test that view. The point of his later statement quoted by Fell and Sauro (that an observation must be for or against some view if it is to be of service) is, of course, that his observations would have been merely another interesting collection of traveller's tales if they had not led him to generalize as to their meaning. Some years after having been made in innocence of any hypothesis, they became important through their role in the generation of a supremely important hypothesis.

The more truly innovative a scientific advance, the more likely that it was based on observations that were, in Fell and Sauro's words, uncontaminated by theory or hypothesis. The basic concepts of metabolic regulation, the area of direct interest here, were formulated in the late 1950s by Umbarger and Pardee. Yates & Pardee (1956) were not thinking about regulation, but merely studying the properties of an enzyme — making observations and not testing any hypothesis. They recognized the existence of feedback regulation because they observed unexpected properties for which an explanation had to be sought. Umbarger (1956) performed his crucial experiments on the basis of a hypothesis, but that hypothesis was

based on observations that had been made by others with no guiding hypothesis in mind.

The reason that these considerations are relevant to our discussion is that Darwin's comment has an equally cogent converse. Science advances through the interplay between observation and generalization. Just as observations are important mainly because they suggest, support, or refute generalizations, generalizations are important only when they relate and rationalize observations and fit them into meaningful conceptual patterns. Observations that are not directly relevant to any existing generalizations are still potentially important — they reflect aspects of reality that require explanation, and they may sometime, like Darwin's initially puzzling observations in South America, be instrumental in the origin of a generalization. But a generalization unrelated to observations or inapplicable to accumulated observations is not useful and is unlikely ever to become so.

The treatments of Kacser & Burns (1973) and Heinrich & Rapoport (1974) [as well as that of Newsholme (Crabtree & Newsholme, 1987), which has received less attention in the symposium, though it is included in the comparison described by Groen and Westerhoff in Chapter 6 of this book] are not based on observation, as are normal scientific hypotheses. In that respect they resemble the *de novo* hypotheses imagined by Popper. Practising metabolic biochemists, who attempt on the basis of observation, generation of hypotheses, further observation, and modification of hypotheses to understand how metabolic processes are regulated, find it difficult to see merit in an approach that reaches conclusions on the basis of unsupported assumptions.

As noted in my pre-symposium paper, Sauro & Fell (1987) claimed to have shown by use of one of those models that the whole basis of our present understanding of the regulation of biochemical sequences — the result of a large number of observations and considerable thought — is incorrect. The actual situation is that the model of Kacser & Burns (1973), on which their claim was based, cannot by its very nature apply to a feed-back regulated system characterized by circularity of cause-and-effect relationships.

In their pre-symposium paper, Fell and Sauro say that the response of a system flux J to an effector Q acting on an enzyme i is the product of the flux control coefficient and an elasticity coefficient:

$$R^J_Q = C^J_i \varepsilon^i_Q \tag{1}$$

That relationship is said to be true whatever the effect of Q on enzyme i. They explicitly add that it is true when Q affects the Michaelis constant (or $S_{0.5}$ value) of enzyme i. The equation shows that however strong the effect of Q on i (indicated by ε^i_Q), it can have no effect on flux through the system if the flux control coefficient (C^J_i) is zero, since then the product R^J_Q must be zero.

Any argument based on statements such as "The equation shows..." should be suspect, since if an equation is invalid or inapplicable the things that it shows may well be erroneous. As follows from discussion earlier in this paper, the trouble with eqn. (1) is that it does not take into account cases in which not only is J a function of Q, but Q is also a function of J. That is the situation for any feedback regulated system, whether technological or biological. A mathematical treatment based on the implicit assumption of one-way interactions between the terms in eqn. (1) cannot be validly applied to a situation characterized by circular cause-

and-effect relationships. We have seen that in feedback-controlled systems the flux control coefficient of every element of the system is zero, yet one of them controls the flux as a consequence of its response to a feedback signal. Probably Fell and Sauro would not have said so confidently that my comment that an enzyme with a flux control coefficient near zero could exert 100% control of flux "cannot be true" if they had considered analogous technological cases such as temperature control in waterbaths, residences, or refrigerators. The lack of correlation that I noted between flux control coefficients and actual control of flux, which they believe to be impossible, is universally true in feedback-regulated systems. It is admittedly inconsistent with their equation, but that is rather different from being impossible.

Salter and Knowles. In their thoughtful comment, Salter and Knowles call for a theory that will relate control coefficients to regulatory importance of enzymes. In spite of their recognition that the flux control coefficient has not been related to control of fluxes, they implicitly assume that such a relationship exists when they say that if the flux control coefficient of an enzyme were 0.0001 it could not contribute to regulation of flux. They fail to recognize that it is in principle impossible for flux control coefficients to be related to flux in most real metabolic systems because the mathematical approach on which the control coefficient is based is unable to deal with the kind of circular causality that is essential to feedback regulation. In consequence their laudable goal of formulation of a theory to relate control coefficients and regulatory importance of enzymes is, I believe, in principle unattainable. As noted above, a flux control coefficient of, or close to, zero is a prerequisite for feedback regulation and it is at feedback-modulated enzymes that regulation is exerted; hence there cannot be a positive relationship between that coefficient and the regulatory importance of an enzyme.

Hofmeyr. Hofmeyr argues that order of reaction is not all that meaningful in our consideration of the enzymes that are responsible for metabolic regulation, because, he says, the kinetic order of any reaction, regardless of the properties of the enzyme, approaches infinity as the reaction nears equilibrium. The implicit rationale for that statement is that at equilibrium the net rate of conversion is zero, and if a small amount of substrate is added there will be a small net forward reaction. The reaction order is then considered to be infinite on the ground that a change from zero to any finite number is an infinite change. That statement is neither valid nor consistent with chemical practice. The concept of a kinetic reaction order for a net conversion is meaningless. Reaction orders can apply only to reactions, not to the differences between forward and reverse reactions. Metabolic reactions have high kinetic orders only because of the properties of the enzymes that catalyse them, and high order is important because it is an essential prerequisite to sensitive partitioning at branch-points.

I must disagree with some other aspects of Hofmeyr's comments, but only one point will be noted. He observes that the mathematical models under discussion include among their assumptions the postulate that the concentration of the terminal metabolite is "clamped by the environment of the system." That assumption begs the question. When we study metabolic regulation, the problem of importance is how concentrations and fluxes are regulated. Our aim must be to increase our understanding of the interactions that underlie

phenomena of interest, and understanding cannot be advanced by excluding important aspects of the systems that we are studying from scientific attention by attributing them to "the environment" or to Mother Nature. An approach to metabolic regulation that considers one of the most important consequences of regulation (the fact that fluctuations in end product concentrations are small as compared to the range of flux values) to be fixed by some mysterious force extraneous to our concerns cannot help us in understanding how those consequences actually arise. It may be intellectually interesting to assume constant concentrations of the first and last substances in a reaction sequence, and then to set oneself formal mathematical problems as to how the rates of reactions in such an artificial system might be affected by changes in various formal parameters. However, that approach severs all connection with the questions that need to be answered if the regulation and correlation of metabolic processes are to be understood. Such an approach is closely related to the failure to recognize the fundamental difference between the demand for the regulatory end-product, which is determined by other metabolic needs, and the interactions within the feedback loop, by which fluxes are adjusted appropriately to meet that demand, while the concentration of the end-product is held within relatively narrow limits.

These are not criticisms specifically of Hofmeyr's comments, since he accurately reflects the assumptions of the mathematical treatments under discussion. But the importance of the distinction between the two approaches — on the one hand, trying to devise experiments to elucidate the regulation of concentrations and fluxes in real cells; on the other, fixing concentrations by edict — cannot, in my opinion, be overemphasized. It is crucial to the question of whether we are to deal with biology on its own terms or with mathematical abstractions, simplified by biologically inappropriate assumptions whenever that is mathematically convenient.

Recapitulation

In conclusion, I must present what I believe to be the answers to the questions posed by Dr. Cornish-Bowden.

(a) *What characteristics should a theory have if it is to be useful to experimentalists?* My answer is that, like generalizations in other fields, it should correlate and rationalize observations and be capable of generating predictions. Thus it must deal directly with the subject matter of metabolism and its regulation. If it is instead a method for handling results, like statistical techniques, it should be applicable to the study of biochemical sequences, and should aid in the design of efficient experiments and the evaluation of the significance of their results.

(b) *Are the main treatments that have been discussed here valuable to experimentalists?* I think the answer must be that they are not. Lacking specific biochemical content, they cannot rationalize biochemical observations and generate predictions. As methods for handling data, they might be useful in dealing with unregulated sequences of reactions, where the properties of the individual enzymes collectively determine the flux. But they are not applicable to regulated sequences or systems, either biological or technological. It is the essence of

regulation that flux responds to a signal, and that it must be essentially independent of the properties of individual components of the system. What is required is that the capacity of each component be larger than any flux that will be called for by the signal so that the system becomes "transparent;" flux is determined only by the signal. Those considerations apply whether the signal is external or internal. When the signal is internal, as in feedback-regulated systems, the relationships become more complex, since causality is circular in such systems, but it is still an essential feature that flux must be nearly independent of the capacities of the individual enzymes and must depend almost entirely on the regulatory signal. In both types of systems (those responding to external or to internal signals), the effectiveness of regulation is related to how nearly total is the dependence of flux on the signal, and hence on how nearly independent the flux is of the capacities of components of the system. Thus mathematical treatments that are based on the response of flux to change in enzyme activity (or to changes that can be expressed as changes in activity) cannot apply to feedback-regulated systems. In searching for usefulness for such treatments, one must look elsewhere than at metabolic regulation, which is exerted primarily through responses to product negative feedback and other types of signalling.

REFERENCES

Crabtree, B. & Newsholme, E. A. (1987) *Trends Biochem. Sci. 12,* 4-12
Heinrich, R.& Rapoport, T. A. (1974) *Eur. J. Biochem. 42,* 107-120
Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27,* 65-104
Sauro, H. M. & Fell, D. A. (1987) *Biochem. Soc. Trans. 15,* 234-235
Umbarger, H. E. (1956) *Science 123,* 848
Yates, R. A. & Pardee, A. B. (1956) *J. Biol. Chem. 221,* 757-770

NOTE ADDED IN PROOF

I thank Dr Kacser for sending me a copy of his urbane and collegial letter (Appendix A in this book) and affording me an opportunity for a brief rejoinder. Dr Kacser's letter does not address a central question — can the models discussed in this book deal with negative-feedback-controlled systems in which no concentrations or fluxes are fixed, and in which the flux through the system and the concentration of the end product are mutually dependent? (It is acceptable, in considering a given regulatory segment of a metabolic system, to assume the concentration of the first or branch-point metabolite to be effectively constant, since that concentration is stabilized by homeostatic interactions that are prior and external to the system under consideration).

In my model, and in actual synthetic sequences, the properties of enzyme E_3 are not relevant. The feedback system responds to the concentration of X_3 in such a way as to minimize variation in that concentration. It thus automatically adjusts the flux through the regulated segment symbolized by E_0, E_1, and E_2 to equal the net rate at which X_3 is removed. The system is oblivious to the properties of the various enzymes that are jointly symbolized by E_3; its response to the rate at which X_3 is removed is indirect, mediated by changes in the concentration of X_3, which is the variable that is sensed.

Appendix A

Edinburgh, 25th September 1989

My dear Dan,

Since you raise questions, of a general nature and of a methodological (and perhaps even of a philosophical) kind, I would like to make some remarks which are not specifically dealt with in my contribution (nor, I imagine, in those of others). I agree with some of the ideas you present, particularly about "understanding" as the aim of science. I am, however, less pessimistic than you and believe that our apparent differences *are* reconcilable. Some of your views, I believe, are due to misunderstanding of what we try to do and also what we are actually doing.

As you rightly say, qualitative questions have logical priority over quantitative ones. All the qualitative questions which you mention are what we usually describe as the "structural" aspects of metabolism — the metabolic map, the positive and negative effectors that act, the branch points, the cycles etc. This is the molecular anatomy with all its connections, but an organism (or even part of one) also has physiological properties. These raise quantitative questions to which we must give quantitative answers. Control analysis attempts to do just that. It does not ask: "is there a feedback from A to B"? but "*if* there is one, what quantitative contribution does it make to the flux in the pathway or the concentration of the metabolites when it is embedded in the rest of the 'structure' of which it is only a part". If you address a *particular* question then, of course, you must use the *particular* quantitative information that you have. But before you can apply it and interpret the measurements, you have to describe your problem in quantitative terms. And here we must use the only quantitative language we know: mathematics. After all, one of the great advances in metabolism was the formulation of the Michaelis-Menten equation. It lacks, as you put it, "specific substantive content". It applies to *any* enzyme (or more complex formulations invented since) or to non-enzymic events. It is only when you put *particular* values of V_{max}, K_m etc. for a *particular* enzyme that it becomes "biochemical". Before that it is simply a hyperbolic equation. Control analysis is no different, except that it deals with *systems* of enzymes instead of single ones. Just as the Michaelis-Menten equation tells you what experiments to do and how to treat the measurements you obtain in order to make quantitative statements about the enzymes, so control analysis tells you what kind of experiments to do (and they *are* different from those on single enzymes) and what to measure under what circumstances. The interpretation of these measurements then yields the quantitative values of the elasticity and control coefficients. One of the important results of this treatment has been to generate theorems (or properties) of *systemic* behaviour and not merely those of its parts. The answer to your question: "can they aid our experimentalist in designing experiments?" is an emphatic YES. Indeed, an ever increasing number of publications by experimentalists testifies to its "usefulness" in understanding how metabolism works.

Why would Groen and his colleagues have "titrated" mitochondria with a number of inhibitors if control analysis had not told him (*a*) to do the titrations and (*b*) to use these measurements to gain insight into the control of oxidative phosphorylation? Why would Stitt and his colleagues have used mutants of *Clarkia* if control analysis had not told him (*a*) to combine these mutants in various ways and (*b*) use measurements on such strains to gain quantitative insight into the control of starch and sucrose synthesis? Why would Salter and his colleagues have used hormones and inhibitors on hepatocytes if control analysis had not told him (*a*) to do such experiments and (*b*) how to use the quantitative analysis of his measurements to gain insight into aromatic catabolism? Why did we ourselves construct heterokaryons in *Neurospora* or use mutants of *Drosophila* to make measurements, to reveal important biological insights into metabolism? Why do so many other workers do these new kinds of experiments? So you are wrong in stating that our approach does not tell experimentalists what to do. Indeed, one of the aims of control analysis is to make people do experiments, as is abundantly clear from our first (and subsequent) publications. These experiments are, however, of a different kind from the "grind and find" which so much of biochemistry (and molecular genetics) consists of.

Whether the results of such experiments and their interpretation are useful (or even true) is an entirely different matter. Because a lot of people do it, does not prove it is right. After all, we had been burning witches for centuries. I will therefore have to convince you, as the Grand Inquisitor, that you are in error in rejecting the new heresies. You try to make your points with reference to a feedback system and your refrigerator. Let me try to tell you where your analysis is wrong and where you misinterpret what we say and do.

Now, Dan, you have seriously misunderstood one of the essential features of our model. We do *not* clamp the concentration of the signal but something *outside* the system (your ambient temperature, for example). The internal temperature, then, is one of the variables. The existence of a feedback will reduce (but not abolish) changes in the internal temperature when some parameter is changed (say the number of openings). When drinking beer such small changes don't matter. But there is one further difference between a thermostat and an enzyme feedback. The thermostat is mechanically designed to "click" on and off. Enzymes respond by a continuous function of the *concentration* of signal. If there were no concentration change in the signal, there could be no response of the rate. This response is measured quantitatively by the feedback elasticity in our control equations. If such feedback occurs at "the first committed step" or at a branch point, then this will be reflected in the structure of our equations. If it is of "high kinetic order", then the feedback elasticity coefficient will be a large negative number. The *mechanism* of feedback action may well be via an alteration in affinity. The *magnitude* of this coefficient will have to be determined experimentally on the enzyme, but its systemic effect — which is all we want to know about — will have to be measured in the whole system. This involves all intrinsic and extrinsic factors and measurements of all the control coefficients. The summation of these to unity is an algebraic certainty and has been confirmed experimentally by Torres *et al.* [*Biochem. J. 234,* 169-174 (1986)] and others[1]. A feedback cannot be said to "control the flux". The immediate effect of the negative feedback by the signal on to an early enzyme is to *reduce* the variation of the concentration in response to *changes in the activities of enzymes*

[1] An extension of the summation theorem to deal with enzyme-enzyme interactions is presented in Chapter 20 of this volume.

proximal to the signal and to changes in the concentration of the "external" substrate to the pathway. This increased "buffering" of the signal will also result in increased buffering of the flux through the pathway *with respect to these signals.* When the feedback is very effective, the signal (and flux) will be substantially constant over a range of enzyme variation. In control analysis terms, this means that the control coefficients $C^J_{e_i}$ and $C^S_{e_i}$ will be very small (where e_i are any of the enzymes proximal to the signal). At the same time, and as an automatic outcome of such a situation, the enzyme(s) distal to the signal will have very high flux control coefficients $C^J_{e_j}$. In the limit as $C^J_{e_i} \to 0, C^J_{e_j} \to 1$. This means that with very effective feedback, most of the control is thrown onto the distal enzymes. Changes in these (changes in "demand") will then "control" the flux in the sense that it will be possible to "extract" a wide range of fluxes almost proportional to the distal enzyme activities (i.e. $C^J_{e_j} \approx 1$) with very little change in the concentration of the signal. This, then, is how a feedback system works: control by the distal enzyme(s) and little control by the proximal enzymes (in the loop and proximal to it). This conclusion cannot have been derived by the closest inspection of the feedback loop itself and its mechanism. It is only by considering its role in the whole system, i.e. control analysis, that this insight is gained.

In your feedback models you appear to fix the output rate, v_3, i.e. the rate is independent of anything proximal to it. For some reason, however, you did not include in the summation the term corresponding to this enzyme, E_3. It is, of course, clear that *all* rates must be included, constant or not. Since you found that all other coefficients were approximately zero, you would have found the coefficient for E3 ($C^J_{v_3}$) approximately unity. So there is no conflict between your simulation and our treatment.

The "link" between control analysis and "regulation" (as traditionally understood) has been presented by Herbert Sauro in his contribution (Chapter 17) to this volume. It is well worth considering.

So I would commend to you (and others of the same persuasion) to look at the little bit of algebra which underlies our approach and note its translation into experiments. That is how control analysis started and where it remains: a method of doing experiments to gain an understanding of all the complex interactions of metabolism.

I have a friend who has a refrigerator. He started off as a bachelor. He filled his refrigerator once a week with beer cans and, every evening, took out three and drank them. He is an ingenious fellow as well as a bit of a miser. He wanted to know how much his beer really cost him. So he fitted up a simple ammeter connected to the compressor and noted the measurements. This gave him the flux (of electricity), F, and hence the cost averaged over a week. He did not like the result. So he reasoned that with better insulation his beer would cost less. He fitted polystyrene panels all round and watched his ammeter and recorded the results., finding that F decreased and approached a small value as the thickness I of the insulation increased. At each point he calculated the cost of adding more insulation, i.e. $\partial F/\partial I$, or to make it independent of the inches and amps, invented a coefficient (which was negative):

$$\frac{\partial F}{\partial I} \times \frac{I}{F} = C^F_I$$

He noted the change in the C value at each point.

He also noted that the kitchen was rather warmer than other rooms, so he tried various positions in the house and found that F increased with the ambient temperature. Next, he

changed the setting of the thermostat (knowing all about Newton's laws of cooling?) and found that F decreased as the thermostat was set to higher temperatures.

He had changed the "control function" (what he later called the "feedback elasticity coefficient"). He had, of course, installed a recording thermometer in the refrigerator and noted that, in the previous two experiments (insulation and ambient temperature) the average internal temperature had changed — decreasing as the amount of insulation increased, but increasing as the ambient temperature increased — although he had not touched the thermostat.

He complained to the shop but they tested the thermostat and found nothing wrong with it. He therefore concluded that the internal temperature, although acting as a signal to the thermostat function, was in fact a variable just as the flux was. They were interdependent and both depended on all the parameters which he could alter.

Then he got engaged to a very nice girl and brought home some chilled TV dinners to entertain her. His flux shot up and he realized that he was opening the door more frequently. So he installed a recorder for the number of times and the length of opening. Again, both flux and internal temperature changed.

Then the inevitable happened and he married the girl. She removed most of the beer and filled the refrigerator with whatever wives fill it with. The ambient temperature in the kitchen went up because she loved baking and the door was never long closed. The flux and the internal temperature went up and so did the temperature in the ice compartment (where they kept the frozen peas and the ice-cream).

He despaired a little of fully understanding what was going on until he happened to read a paper, published in 1973, which suggested he was dealing with system properties and not just the nature of the thermostat. In this paper there was an explicit treatment of a feedback system. One thing, though, worried him. The paper was all about Biochemistry yet he was dealing with a refrigerator.

Eventually he realized that the formal descriptions of the two systems were almost the same but that they obtained their "biochemical" or "refrigerator" meaning by identifying the parameters and the variables. He learnt that in both cases large numbers of parameters were responsible for determining the variables, that the average internal temperature was not fixed by the thermostat but depended on the nature of its control function as well as on the quantitative values of other features of the box. It was only by measuring the effects of variation in all of them that he obtained a complete understanding. He has lived happily ever after, drinking less beer, eating better meals and paying more for his electricity.

Yours sincerely,

H. KACSER

Appendix B

Computer Programs

THE PRIMARY purpose of the methods of analysis described and developed in this book is to promote understanding of the ways in which fluxes and concentrations of metabolites are controlled in biochemical systems. The capacity to reproduce the behaviour in the computer is hardly essential for understanding the ideas set out in the various chapters, but it is certainly very helpful, because it allows one to translate the "thought experiments" that are often used in studies of metabolic control into experiments *in numero:* provided one can specify the system under discussion precisely enough one can determine in the computer whether it really does respond to influences in the way that one predicts. Suitable programs are tedious and difficult to write, but easy for anyone with a basic understanding of kinetics to use. We therefore note here the existence of some programs that are currently available for modelling metabolic systems in the computer.

CONTROL

Available from:	T. Letellier and J.-P. Mazat, Département de Biochimie, Université de Bordeaux II, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France.
Cost:	None.
Documentation:	Notice in French. A short notice in English will soon be available.
Hardware requirements:	IBM PC, XT, AT or true compatible. A printer is useful but not necessary.
Limitations:	CONTROL can accept up to 19 reactions and 19 metabolites.
Language:	CONTROL is written in Turbo Pascal 3.0.

CONTROL uses the method developed by C. Reder [*J. Theor. Biol. 135,* 175-201 (1988); also pp. 77-96 in *Contrôle du Métabolisme Cellulaire* (Mazat, J.-P. & Reder, C., eds.), Bordeaux, 1988]. It can be applied to *any metabolic network* (linear, branched, with substrate cycles, etc.) and is intended for (i) determining the flux and concentration control coefficients from the elasticity coefficients, and (ii) writing formally the summation and connectivity relationships (for flux and concentrations). It only requires the writing of the stoicheiometry matrix of the metabolic network (the **N** matrix in the above references).

ESSYNS — Evaluation and Simulation of SYNergistic Systems, Version 2.00

Authors:	Douglas H. Irvine, Michael A. Savageau and Eberhard O. Voit.
Available from:	Douglas H. Irvine, Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347, USA.
Cost:	US $40.00 for program disks and printed documentation.
Documentation:	137-page *User's Guide to ESSYNS* by Eberhard O. Voit, Douglas H. Irvine and

Michael A. Savageau, plus 31 on-line help menus.

Hardware requirements: IBM PC, XT, AT, PS/2 or compatible computer with 512K of memory. Graphics require a CGA, EGA, VGA, Hercules, ATT, PC3270 or IBM8514 video card; a colour monitor is not required but can be used for special effects. Graphics can be printed directly on an Epson, Okidata or IBM dot-matrix printer. A math co-processor is not required but is used when available for faster numerical computation. Extended memory (EMS) also is not required but is used when available for faster operation.

Limitations: Up to 25 simultaneous non-linear ordinary differential equations expressed in *S*-system canonical form. Solutions have up to 15 significant digits.

Language: Borland Turbo Pascal 5.0.

ESSYNS computes dynamic solutions for complex non-linear systems, as well as eigenvalues and full steady-state sensitivity profiles. Results can be displayed or printed in tabular format, or they can be displayed or printed in two- or three-dimensional graphs. Use of the program does not require any knowledge of the underlying mathematical details. No programming or compiling is required, and all options in the program are selected with simple, single-letter commands. A new variable-order, variable-step Taylor-series method has been specially developed for ESSYNS to maximize its efficiency and accuracy [Irvine & Savageau, *SIAM J. Numer. Anal.* in press (1990)]. For example, on an 8MHz PC-compatible computer with a math co-processor, a representative problem (described in the article cited above) requires only 0.87 second to generate a complete dynamic solution with more than 300 points accurate to six significant digits.

GEPASI

Available from: Pedro Mendes, C.E.B.F.A., Cç Bento Rocha Cabral 14, 1200 Lisboa, Portugal.

Cost: US $35.00 for a registered copy with printed manual and tutorial. Sharing of copies on a shareware basis is allowed.

Documentation: Printed manual with tutorial for registered users. Text file with quick user guide is included in the disk.

Hardware requirements: IBM PC, XT, AT, PS2 or true compatible with one disk drive and at least 256 kilobytes memory. A VAX/VMS version is also available (write for details).

Limitations: GEPASI can only handle pathways with uni-uni enzymes and accepts up to 20 enzymes and 20 pools (either internal or external). Many reaction kinetics available!

Language: GEPASI is written in C and compiled with Microsoft C 5.0 (or with the VAX/VMS C compiler).

GEPASI is a program that simulates the dynamic behaviour of metabolic pathways. It uses a macroscopic approach to the kinetics of each enzyme in the pathway as described in Chapter 16 of this book. The user must define the pathway structure and kinetic parameters as well as metabolite initial concentrations in an ASCII file and GEPASI outputs the concentrations of all metabolites as a function of time. It also calculates the elasticities and control coefficients at the steady state. The package includes a special program for interactive build-up of input files, ready-to-run examples and a program for graphical display of results (works with all common graphics cards).

GEPASI does not make approximations to the dynamics of the pathways. As long as the kinetics of each enzyme is really described by the mathematical model chosen the dynamics will be just like the simulation (if other effects such as temperature, pressure etc. are negligible). Moreover, simulations can be taken in large finite time intervals. GEPASI accepts any pathway that has up to 20 metabolites and 20 enzymes. Eleven different kinetic models can be chosen for each enzyme. The structure can include branches (even with more than three arms) and substrate cycles (with two enzymes or longer) as long as the limits above are not exceeded. No mass conservation cycles can be included just because the current version of GEPASI does not accept bimolecular reactions.

Registered users will be notified of future versions of GEPASI that will be available at very reduced prices (mainly postal charges).

METAMOD

Available from: J.-H. S. Hofmeyr, Department of Biochemistry, University of Stellenbosch, Stellenbosch, South Africa 7600; but *see NOTE ADDED IN PROOF below.*

Cost: Equivalent of US $10.00 to cover mailing and photocopying costs.

Documentation: A user's manual and a file READ.ME that contains the latest information is provided.

Hardware requirements: IBM PC, XT, AT or true compatibles with at least 512 kilobytes of memory. Since there is no graphical output, any graphics card will do. A mathematical co-processor will be sensed automatically but is not essential. The original version written in Basic for the BBC Microcomputer (see reference below) has not been updated.

Limitations: The size of the metabolic pathway depends on the amount of memory available. Typically, 15 reactions; 20 metabolites and 5 conservation equations are allowed.

Language: METAMOD is written in Turbo Pascal 5.0.

METAMOD [*Comp. Appl. Biosci. 2,* 243-249 (1986)] is a tool designed to help students, teachers and researchers explore the behaviour and control of metabolic steady states. Its use is interactive, easy to master and requires no knowledge of programming. Metabolic pathways are defined in terms of reactions, rate equations and conservation equations; METAMOD calculates the steady state by solving the balance equations directly and calculates the elasticity and control coefficients by finite difference methods. When run on a 8 MHz IBM XT without 8087 co-processor the pathway shown in Fig. 1 of the above paper (6 variable intermediates, 8 reactions) reached steady state in 45 s.

NOTE ADDED IN PROOF. METAMOD has now been merged with MetaModel (next entry) and will not be maintained in the future as a separate program.

MetaModel

Available from: Athel Cornish-Bowden, CNRS - CBM2, 31 chemin Joseph-Aiguier, BP 71, 13402 Marseille, France.

Cost: US $25.00 or 100 FRF to cover mailing and copying costs (US price includes bank charges). It may be transferred freely for any non-commercial purpose.

Documentation: Printed manual with up-date information in a file on the disk.

Hardware requirements: IBM PC, XT, AT or true compatibles with at least 512 kilobytes of memory.

Limitations: Systems of up to 15 reactions involving up to 20 metabolites can be handled.

Language: MetaModel is written in Turbo Pascal 5.0.

MetaModel was originally written in Fortran for teaching the principles of control analysis to undergraduates with little or no prior experience of computers and no prior knowledge of control analysis. It was designed to allow such users to enter their own pathways and study their steady states within an hour or two of first encountering the program. The current version has been completely rewritten to take account of the different characteristics of PCs as compared with main-frame computers, but it retains the easy-to-use features. The menu structure is designed to provide ample help to first-time users while allowing more experienced users to avoid endlessly repeating the same inputs. MetaModel does not provide graphical output directly, but produces output files that can be read and processed by commercial spreadsheet programs (Quattro, Lotus 1-2-3, etc.)

Modelling Metabolism on an Electronic Spreadsheet

Available from: D. E. Atkinson, Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024, USA.

Cost: None.

Documentation: Instruction sheet supplied with the disk.

Hardware requirements: Macintosh Plus or higher with at least 1 megabyte of RAM.

Other software needed: Excel.

Limitations: Simulation of a specific situation; not a general model.

Language: Excel.

This model is implemented in Excel, one of several easy-to-learn, non-procedural computer languages known as spreadsheets. It is based on the general principles set out in *Dynamic Models in Biochemistry* by D. E. Atkinson, S. G. Clarke, D. C. Rees, and D. S. Barkley (N. Simonson & Company, 13450 Maxella Avenue, Marina del Rey, California 90292), a spreadsheet workbook for undergraduate students that is intended to put simple biochemical simulation within reach of users with no knowledge of formal computer programming. The present model is specific rather than general. It simulates a three-enzyme biosynthetic sequence, the first enzyme of which is regulated by endproduct negative feedback. Within that limited compass, all relevant kinetic parameters, including the degree of cooperativity and the strength of the feedback effect, are assignable.

✂ ---

ORDER FORM

To *(enter name and address as shown above)*:

Name of program:

Computer to be used (enter IBM PC, AT, Macintosh Plus etc.):
Check Hardware Requirements listed above before ordering

Disk reader: 5.25 inch/3.5 inch
Strike out inapplicable entries

Enclosed: Blank disk 5.25 inch/3.5 inch + cheque/money order for _____
Strike out inapplicable entries *Enter amount*

Program to be sent to:
Enter name and address and enclose pre-addressed label

Signature: _____ Date: _____

Contributors

LUIS ACERENZA • Department of Genetics, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK

KATHY R. ALBE • Division of Biological Sciences, University of Montana, Missoula, Montana 59812-1201, USA

DANIEL E. ATKINSON • Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024, USA

MICHAEL N. BERRY • Department of Medical Biochemistry, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042 Australia

JEAN BUC • Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

ENRIC I. CANELA • Departament de Bioquímica i Fisiología, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Catalunya, Spain

MARÍA LUZ CÁRDENAS • Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

MARTA CASCANTE • Departament de Bioquímica i Fisiología, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Catalunya, Spain

P. BOON CHOCK • Laboratory of Biochemistry, National Heart, Blood and Lung Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

ATHEL CORNISH-BOWDEN • Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

ANTONY M. DEAN • Department of Biochemistry, University of California, Berkeley, California 94720, USA

JOHN S. EASTERBY • Department of Biochemistry, The University, P. O. Box 147, Liverpool L69 3BX, UK

RONALD D. EDSTROM • Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455, USA

D. FENNELL EVANS • Center for Interfacial Engineering, University of Minnesota, 435 Delaware S.E., Minneapolis, Minnesota 55455, USA

DAVID A. FELL • School of Biological and Molecular Sciences, Oxford Polytechnic, Oxford OX3 0BP, UK

RAFAEL FRANCO • Departament de Bioquímica i Fisiología, Universitat de Barcelona, Martí i Franquès 1, 08028 Barcelona, Catalunya, Spain

CHRISTOPH GIERSCH • Universität Düsseldorf, Institut für Biochemie der Pflanzen, Universitätsstraße 1/26.03, D-4000 Düsseldorf, Federal Republic of Germany

ALBERT GOLDBETER • Faculté des Sciences, Université Libre de Bruxelles, Campus Plaine, C.P. 231, B-1050 Brussels, Belgium

ROLAND B. GREGORY • Department of Medical Biochemistry, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042 Australia

ANTHONY R. GRIVELL • Department of Medical Biochemistry, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042 Australia

ALBERT K. GROEN • Department of Gastroenterology, FO-116, Academisch-Medisch Centrum, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

MARY E. GURNACK • Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455, USA

M. HANLON • Department of Medicinal Chemistry, Burroughs Wellcome Company, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709, USA

REINHART HEINRICH • Humboldt-Universität zu Berlin, Sektion Biologie, Bereich Biophysik, Invalidenstraße 42, 1040 Berlin, German Democratic Republic

DEBRA C. HENLY • Department of Medical Biochemistry, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042 Australia

BENNO HESS • Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddamm 201, 4600 Dortmund 1, Federal Republic of Germany

JOSEPH HIGGINS • Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

JAN-HENDRIK S. HOFMEYR • Department of Biochemistry, Faculty of Natural Sciences, University of Stellenbosch, Stellenbosch 7600, Republic of South Africa

DOUGLAS H. IRVINE • Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347, USA

HENRIK KACSER • Department of Genetics, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK

TAMÁS KELETI • Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest P O Box 7, Hungary

NICOLAS KELLERSHOHN • Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

RICHARD G. KNOWLES • Department of Biochemical Sciences, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

D. E. KOSHLAND, JR. • Department of Biochemistry, University of California, Berkeley, California 94720, USA

JOHANNA G. KOSTER • Hubrecht Laboratory, Utrecht, The Netherlands

DIRK LÄMMEL • Universität Düsseldorf, Institut für Biochemie der Pflanzen, Universitätsstraße 1/26.03, D-4000 Düsseldorf, Federal Republic of Germany

T. LETELLIER • CNRS-ICBN, Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France

MARIO MARKUS • Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddamm 201, D-4600 Dortmund 1, Federal Republic of Germany

JEAN-PIERRE MAZAT • CNRS-ICBN, Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France

MARILYN H. MEINKE • Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455, USA

ENRIQUE MELÉNDEZ-HEVIA • Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de la Laguna, 38206 Tenerife, Canary Islands, Spain

PEDRO JOSÉ P. MENDES • Centro de Estudos de Bioquímica e Fisiologia Animal, Instituto Rocha Cabral, Calçada Bento da Rocha Cabral 14, 1200 Lisboa, Portugal

JOÃO MONIZ BARRETO • Centro de Estudos de Bioquímica e Fisiologia Animal, Instituto Rocha Cabral, Calçada Bento da Rocha Cabral 14, 1200 Lisboa, Portugal

R. MULLIN • Department of Medicinal Chemistry, Burroughs Wellcome Company, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709, USA

JUDIT OVÁDI • Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest H-1502, Hungary

JOHN W. PHILLIPS • Department of Medical Biochemistry, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042 Australia

CHRISTOPHER I. POGSON • Department of Biochemical Sciences, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

ANA PONCES FREIRE • Centro de Estudos de Bioquímica e Fisiologia Animal, Instituto Rocha Cabral, Calçada Bento da Rocha Cabral 14, 1200 Lisboa, Portugal

JOHN W. PORTEOUS • Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, UK

CHRISTINE REDER • UER de Mathématique et Informatique, Université de Bordeaux I, 351 cours de la Libération, 33405 Talence Cedex, France

SUE GOO RHEE • Laboratory of Biochemistry, National Heart, Blood and Lung Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

JACQUES RICARD • Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

KENNETH E. RUDD • Division of Bacterial Products, USFDA, Bethesda, Maryland, USA

MARK SALTER • Department of Biochemical Sciences, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

HERBERT M. SAURO • Department of Genetics, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh EH9 3JN, UK

MICHAEL A. SAVAGEAU • Department of Microbiology and Immunology, The University of Michigan, Ann Arbor, Michigan 48109-0620, USA

J. RANKIN SMALL • Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

GARY K. SMITH • Department of Medicinal Chemistry, Burroughs Wellcome Company, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709, USA

JEAN-MICHEL SOULIÉ • Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, 13402 Marseille, France

EARL R. STADTMAN • Laboratory of Biochemistry, National Heart, Blood and Lung Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

KLAUS STEFFEN • Mathematisches Institut der Universität Düsseldorf, Universitätsstraße 1/25.13, D-4000 Düsseldorf, Federal Republic of Germany

DAVID M. STEINHORN • Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota 55455, USA

MARK STITT • Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth, 8580 Bayreuth, Federal Republic of Germany

NÉSTOR TORRES • Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de la Laguna, 38206 Tenerife, Canary Islands, Spain

MARIELLE VAN WORKUM • Division of Molecular Biology, Netherlands Cancer Institute H5, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

ANA VAZ GOMES • Centro de Estudos de Bioquímica e Fisiologia Animal, Instituto Rocha Cabral, Calçada Bento da Rocha Cabral 14, 1200 Lisboa, Portugal

EBERHARD O. VOIT • Department of Biometry, Medical University of South Carolina, Charleston, South Carolina 29425-2503, USA

PATRICIA G. WALLACE • Department of Medical Biochemistry, School of Medicine, The Flinders University of South Australia, Bedford Park, South Australia, 5042 Australia

G. RICKEY WELCH • Department of Biological Sciences, University of New Orleans, Lake Front, New Orleans, Louisiana 70148, USA

HANS V. WESTERHOFF • Division of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

BARBARA E. WRIGHT • Division of Biological Sciences, University of Montana, Missoula, Montana 59812-1201, USA

RUI YANG • Center for Interfacial Engineering, University of Minnesota, Minneapolis, Minnesota 55455, USA

XIURU YANG • Center for Interfacial Engineering, University of Minnesota, Minneapolis, Minnesota 55455, USA

Index

accuracy, 89-100
  branched pathways, 92-97
  criterion of, 96 (footnote)
  enzyme-enzyme interactions, 98
  importance, 90-92
ACERENZA, L., 251-257, 297-302; 53, 145, 289, 298, 302, 303
activity of enzymes *in vivo,* 317-328
Adair, G. S., 36-37
Adair equation, 36-37
adaptation, molecular, 389-397
additivity, 54, 251, 253-256
adenylylation/deadenylylation cycle, 183-194, 201
aggregation of fluxes, 75, 77, 79-80, 86, 89, 91, 92, 93
  strategy, 97
Ainslie, G. R., 292
ALBE, K. R., 317-328
Alberty, R. A., 71
aldolase, 260, 274, 275-279
allosteric enzymes, 7, 173
  compared with interconvertible enzymes, 176-177, 191-192, 199, 216
  kinetic power, 261-262, 267
Altman, C., 224, 240
amino acid metabolism, 63, 161, 377-384
amphibolic pathways, 84
  accuracy of representation, 97-98
amplification of signals, 187-188
5'-AMP nucleotidase, 320
Anderson, W. W., 184
anti-tumour chemotherapy, identifying target enzymes, 385-387
archaebacteria, 389
arginine synthesis, 63
arom complex of *Neurospora,* 282-283
aromatic amino acid metabolism, 377-384
aspartate aminotransferase, 260, 272-274 (*see also* aspartate transaminase)
aspartate shuttle, 344
aspartate transaminase, 320, 321, 323 (*see also* aspartate aminotransferase)
association-dissociation of subunits, 248, 255, 274
Astumian, D., 191
ATKINSON, D. E., 3-11, 413-427, 435-436; 11-26, 31, 39, 60, 227, 322, 340, 429
ATP-ADP-AMP pool, 4, 423
ATPase, Na/K, 338
ATP requirements in hepatocytes, 345-346
ATP synthase, 344

bacteria, growth of, 63
balloon, 241-242
Barkley, D. S., 436
Barnet, S., 149
Bartha, F., 264, 283
Barthelmess, I. B., 411
Bartrons, R., 60, 159
Barwell, C. J., 281
Batke, J., 260
Beavo, J. A., 173, 176
Benson-Calvin cycle, 294 (*see also* Calvin cycle, 355-361, 363-376)
Bergmann, 71
Berlin Group, 66 (see also Heinrich, R., Rapoport, S. M, Rapoport, T. A.)
Bernhard, S. A., 259, 271, 275
BERRY, M. N., 343-350; 65, 344, 349
Bertalanffy, 71
Bialojan, C., 204-206
bicyclic cascades, 178-180, 192-193, 204
bifurcation points, 309, 334
birhythmicity, 310-311
biochemical systems theory, 31, 33, 66, 69-87, 89, 144
  applications, 83-84
  compared with other theories, 101-118, 145-147, 410-411
  dynamics, 91
  explicit *S*-system representation, 74-76, 78
  generalized-mass-action representation, 76-78, 83
    flux-oriented theory as case of, 78-79, 83
  gene expression and, 410-411
  roots of, 71-72
  system representations, 73-74
  Taylor series in, 72-73
  variants, 73-74, 83, 89
biosensors, enzymes as, 291-296, 312
bisphosphoglycerate mutase, 338

bisphosphoglycerate phosphatase, 338
bistability, 173
Blackman-Putter principle, 45
Bloch, W., 321
Bode, 71
Bode analysis, 72
Bohnensack, R., 63
Boiteux, A., 307
Bontemps, F., 348
Boscá, L., 14
Bossi, L., 408
branched pathways, 15, 60, 84, 141-142, 145-147
  accuracy of different representations, 92-97
  experimental example, 363-376
  summation relationships, 104
branching theorems, 104
branch-point
  regulation at, 4, 9, 10, 16-17, 21, 60
  relationships, 141, 354
Brand, M. D., 63, 64
Brauer, M., 367
Brautigan, D. L., 210
Brezhnev, L., 34
Brindle, K. M., 63
Brocklehurst, K., 263
Brown, A. J., 71
Brown, M. S., 184-185
Brown, R. R., 380
Brumen, M., 159, 334, 337, 338
BUC, J., 291-296; 312
Bücher, T., 267, 344
Burk, D., 261
Burns, J. A.,
  with Kacser (1973, 1979, 1981): *see* Kacser, H.
  with ten others (1985), 26, 103, 112, 197, 297, 307
Burton, A. C., 45
Butler, M. H., 318

Caban, C. E., 189
calmodulin, 211, 260, 277-279
Calvin cycle, 355-360, 363-376 (*see also* Benson-Calvin cycle, 294)
Cameron, G., 149
cAMP, effect on muscle glycogenolysis, 210-212
cancer chemotherapy, identifying target enzymes, 385-387
CANELA, E. I., 149-156, 157-169; 82, 116, 165, 340
carbamoyl phosphate synthetase, elasticity for carbamoyl phosphate, 163
carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), effect on DNA supercoiling, 409
carboxyatractyloside, use in study of mitochondrial respiration, 162
CÁRDENAS, M. L., 195-207; 53, 62, 63, 177, 180, 189, 195, 196, 198, 199, 200, 202, 203, 292, 347
Carlson, G. M., 214
Caron, M. G., 176
cascades, 84
  enzyme, 173-217, 248, 422 (*see* interconvertible enzymes for detailed entries)
  period-doubling, 307
CASCANTE, M., 149-156, 157-169; 82, 116, 144, 149, 152, 159, 163, 252, 340
catalytic power, 261
catalytic/specific effects in interconvertible enzyme systems, 197, 198-202
catastrophe, sensitivity, 359
Cayley, A.,71
Cellular Energy Metabolism and its Regulation (D. E. Atkinson), 17
cellular organization, high degree of, 349
cellular volume, control coefficients, 337-338
chain of local effects, 244-246
Chance, B., 41-43, 45, 46
channelling, 74, 248, 256-257, 259-270, 271-279, 286-289, 321, 344, 345-346, 349-350
  efficiency, 287-289
chaos, 298, 302, 303-313
chemical signals, sensing of, 291-296
chemostat, use for estimating fitness, 390, 393
chemotaxis, 291
chemotherapy, identifying target enzymes, 385-387
Chen, Y.-D., 59, 129, 144, 149, 251, 332, 353, 400, 405
Chernavskij, D. S., 340
Chiang, E., 298
CHOCK, P. B., 183-194; 174, 178-179, 183, 185, 187, 188, 189, 190, 195, 196, 201, 275, 422
Christiansen, J. H., 46
citrate synthase, 260, 319, 320, 321
citric acid cycle, 318-327, 328 (*see also* tricarboxylic acid cycle, 317; Krebs cycle, 61)
citrulline synthesis, 63, 161, 162
Clarke, S. G., 436
Clarkia xantiana, sucrose synthesis in, 363-376, 430
Clegg, J. S., 259, 347
Cleland, W. W., 261
Cohen, P., 173, 176, 178, 196
Cohn, N. K., 386
combined response property, 24, 62, 227
competitive/uncompetitive effects in interconvertible enzyme systems: *see* catalytic/specific effects

complexes (enzyme-enzyme), 255-256, 259-260
computers,
models, 6, 12-13, 35, 41, 42-43, 48
programs, 85-86, 138, 141, 145, 318, 433-436
simulation, 165, 221-224, 284
concentration control coefficient, 157 (*see also* control coefficient)
concentration-control numerator, 241
concentration-control path, 242
conditioned elasticity, 228 (*see also* partitioned regulatory coefficient)
connectivity property, 58-60, 81-82, 104, 140, 158, 251-252, 255, 332, 400, 405, 408-409
conservation relationships and, 143
experimental example, 374-375
geometrical interpretation, 133, 137
Connick, J. H., 381
conservation relationships, 121-123, 143, 144
consecutive reactions, 281 (*see also* coupled reactions)
consumers' test of control theories, 101-118
CONTROL (computer program), 138, 433
control analysis,
amino acid metabolism, 63, 161, 377-384
association-dissociation of subunits, 255
assumptions in classical approach, 251, 352
constraints in application to hepatocytes, 63, 343-350
enzyme-enzyme interactions, 251-257
experimentalist's view of, 413-427
independence from kinetic mechanisms, 256-257
inhibitors, use of, 64-65, 385-387
photosynthesis, 351-361, 363-375
relation to regulation, 21-23, 225-230, 382-383, 409-410, 417
theorems, 331-333, 353
theory of, 53-57
control coefficient, 24, 55, 56-57, 103, 123-125, 225, 226-227, 297, 329, 330, 382-383
concentration (or metabolite), 57, 60, 157, 104, 111
determination, 157-169, 221-224, 351-354
amino acid metabolism, 63, 161, 377-384
combined response coefficients method, 161-162
enzyme titration, 160, 231-238
erythrocyte glycoluysis, 337-338
genetic methods, 159-160, 367-371
hepatocytes, 343-347, 379-381
lactose metabolism, 391-397
in vivo, 317-328
pathway shortening, 231-238
photosynthesis, 357-361, 367-371
flux, 56, 383, 385-387, 399, 395
general relationships, 60
global nature, 157-159
relationship to elasticity coefficients, 57-58, 103, 111, 127, 330, 331
time-dependent, 333-334
uniqueness, 353
control denominator, 241
control hierarchy, 399-405
democratic, 404-405
dictatorial, 401-404
control matrix
gluconeogenesis, 107
structural properties, 125-126, 127
relationship to elasticity matrix, 27
control-pattern analysis, 239-248
control strength, 330 (*see also* response coefficient)
control theories,
comparison, 101-118
metabolic and biochemical content, lack of, 414
controllability coefficient, 61
converging pathway, *S*-system representation, 92, 95-97
cooperativity, 7, 11, 26, 33, 39
apparent, interconvertible enzymes and, 176-177, 188-189
indexes, 176, 197
relationship between, 176, 201, 212
transient time and, 285-286
coordinate control operation, 298-302
CORNISH-BOWDEN, A., 17, 31-40, 195-207, 435
(1979), 51, 299
(1986), 200
(1987), 261, 265
(1989), 31, 96 (footnote), 145
(THIS SYMPOSIUM), 328, 413, 414, 417, 426
with Cárdenas (1987), 292
with Cárdenas (1989), 53, 62, 63, 177, 180, 189, 195, 196, 198, 199, 200, 202, 203
with Ricard (1987), 33
with Storer (1974), 38
with Storer (1976), 39
Corredor, C., 13, 16
coupled reactions, 259-270, 281
covalent modification systems, 173-217 (*see* interconvertible enzymes for detailed entries)
Cox, D. E., 209
Crabtree, B., 5, 18, 20, 33, 40, 69, 78-79, 89, 101, 112-113, 144, 149, 227, 266, 424
Crabtree-Newsholme theory, 40, 111-113, 117, 227, 424 (*see also* flux-oriented theory)

Crick, F., 31
critical slowing down, 303-313
crossover, 51-52
Crozier, W. J., 45-46
Cseke, E., 260
cycle, moiety-conserved, 57, 60-61, 246-248
cyclic cascade mechanism: *see* interconvertible enzymes
cyclic systems, 15
cycling, substrate, 107, 142
cytoskeleton, enzyme association and, 278-279
cytosociology, 259

Dahlberg, D., 318
Dalziel, K., 71
Damjanovich, S., 259
Danforth, W. H., 190
Darwin, C., 13, 32, 423
DEAN, A. M., 389-398; 63, 389-390, 391, 395, 397, 401
5'-deazaacyclotetrahydrofolate, effect on *de novo* purine biosynthesis, 385-387
Decroly, O., 298, 310, 312
Degn, H., 173
de novo purine biosynthesis, 63, 385-387
democratic hierarchy of control, 404-405
democracy, molecular, 259
Demongeot, J., 298
Derr, R. F., 61, 65
Descartes, R., 71
design of pathways, 339-342
development, histones and, 406-407
diarrhoeic shell-fish poisoning, 204
dictatorial hierarchy of control, 401-404
Dictyostelium, 318-328
differential equations, stiff, 223
diffusion-limited processes, 291
dimension, embedding, 306
DiTullio, N. W., 344
diverging pathway, *S*-system representation, 92-95
DNA gyrase, 407-409
DNA/protein ratio in development, 406-407
DNA supercoiling, 407-409
Dobzhansky, Th., 33
Doehlert, D. C., 264
Dombradi, V., 209
dominance, 13
Donnan equilibrium, 336-337
downstream effects, 227-230
Drlica, K., 407
Drosophila, mutants, 430
dual modulation method, 373
Duch, D. S., 386
Duszynski, J., 343-344, 346
Dykhuizen, D. E., 390, 396
Dynamic Models in Biochemistry (D. E. Atkinson *et al.*), 17, 20, 436
dynamics, representation of, 91

EASTERBY, J. S., 281-290; 38, 222, 264, 271, 272, 281, 282, 283, 284, 285, 298, 422-423
Edelson, D., 303
Edelstein, M., 386
Edelstein, S. J., 212
EDSTROM, R. D., 209-217; 178, 209, 214
effectors,
  assessing relative importance, 225-230
  internal, 227
  partially internal, 113
elasticity coefficient, 15, 24, 55-56, 103, 157, 225-226, 317, 330
  absolute, 110
  conditioned, 228 (*see also* partitioned regulatory coefficient)
  control-pattern analysis and, 243-246
  direct calculation, 163
  equivalent to kinetic order, 76
  gluconeogenesis, 105-106
  indirect calculation, 162-163
  kappa (κ) elasticities, 61-62, 63
  local character, 158
  matrix, relationship to control matrix, 127
  "overall", 114
  parameter (π) elasticities, 252-256
  photosynthesis, 370-371, 372-373
  relationship to control coefficients, 57-58, 103, 111, 127, 330, 331
electroconformational coupling, 349
El-Maghrabi, M. R., 189
embedding dimension, 306
endogenous protein as energy source, 318-319
energy charge, 21
energy cost of interconvertible enzymes, 174, 180-181, 190-191
energy state and DNA supercoiling, 407-409
Engasser, J. M., 291
enolase, 344
Entman, M. L., 214
entrainment, 307
enzyme activity *in vivo*, estimation, 317-328
enzyme cascades, 173-217 (*see* interconvertible enzymes for detailed entries)
enzyme concentration, high, 321, 355
enzyme-enzyme interactions, 98, 159, 248, 259-260, 266, 271-279
enzyme titration, 160, 231-238
epidemiology, 84

epilepsy, 381
equilibrium enzymes: *see* near-equilibrium enzymes
erythrocyte metabolism, 109, 329-342
Eschrich, K., 173
ESSYNS (computer program), 85-86, 433-434
Euler, L., 71, 252 (footnote), 354, 355
European Journal of Biochemistry, 48
EVANS, D. F., 209-217
evolution,
  molecular, 389-398
  neutral theory, 389
excluded enzyme, 242
experimentalist's view of control analysis, 413-427
external effectors, 61-62

falsifiability, 32-35, 423
far-from-equilibrium reactions, 52
Farquhar, G. D., 355, 356
FCCP, effect on DNA supercoiling, 409
feedback effects, 47, 51, 151-154, 229-230, 284-285, 301, 385, 387, 416, 419-422
  discovery, 423-424
  numerical simulation, 419-422
feedback repression, 404, 410
feedforward effects, 151-154, 278
Feigenbaum route to chaos, 307
FELL, D. A., 13-15, 139-148
  (THIS SYMPOSIUM), 328
  with Sauro (1985), 58, 60, 82, 104, 117, 129, 138, 141, 143, 144, 149, 158, 221, 239, 252, 255, 256, 354
  with Sauro (1987), 5-9, 10, 14-15, 25-26, 117, 416
  with Sauro (THIS SYMPOSIUM), 39, 423-425
  with Sauro and Small (1987), 60, 221, 239, 255, 256
  with Sauro and Small (1988), 158, 159
  with Sauro and Small (THIS SYMPOSIUM), 61, 221
  with Small (1986) 195, 197
  with Small (1987), 60
  with Small (1989), 60, 142, 144, 158, 239
  with Snell (1988), 139, 140, 159
Ferone, R., 386
Fersht, A., 261
Feynman, R. P., 38
Fick's law of passive diffusion, 392
Figueroa, N., 408
Finney, R. L., 72
fitness
  flux and, 391-395
  measurement of, 390-391
Fitzgerald, T. J., 214
Flint, H. J., 63, 160, 401
Flügge, U.-I., 363
fluid flow, dynamics, 303
flux,
  effect of V_{max} and $S_{0.5}$, 419-427
  fitness and , 391-395
flux control coefficient, 24, 157 (*see also* control coefficient)
flux-control numerator, 241
flux-control path, 241
flux-oriented theory, 69, 78-79, 89-90, 101, 111 (*see also* Crabtree-Newsholme theory)
  compared with other theories, 101-118
formalisms, mathematical, 71-72
Fourier, J., 71
FRANCO, R., 149-156, 157-169; 82, 116, 165, 328, 340
Frank, P. M., 149, 150
frequency mixing (quasiperiodicity), 307
Friedrich, P., 259
Friend, D. S., 344
fructose 6-phosphate 2-kinase, 365, 366 (*see also* 6-phosphofructo-2-kinase, 189)
fructose 1,6-bisphosphatase, 260, 274, 277, 294-296, 364, 365-368
fructose 2,6-bisphosphatase, 189, 365, 366
fructose 2,6-bisphosphate,
  discovery, 32
  regulator cycle, 365, 371-374
fumarase, 321, 322

Gaertner, F., 282, 287
β-galactosidase, 392-397
Galileo, G., 71, 86
Garcia, E., 184, 189
Gardeström, P., 364
Garfinkel, D., 35
Gear method, 223
Geigy, J. R., 98
Gellerich, F. N., 63
Gellert, M., 407, 411
gene dose, change of, 63
gene expression, 63, 178, 399-412
general principles, none in biology, 4
generalized mass action, 74, 76-82, 90
  accuracy, 92-97
  flux-oriented theory as case of, 78-79
  implicit form, 79-81
  metabolic control theory as case of, 81-83
geometrical aspects of metabolic control, 129-138
GEPASI (computer program), 434
Gerhardt, R., 364, 366

Ghazi, A., 396
GIERSCH, C., 351-361; 54, 59, 60, 82, 141, 144, 252, 256, 352, 353, 354, 355, 359
Gifford, R. M., 363
Ginsburg, A., 183, 185, 189
Glaser, R., 337
global properties, 157-159
glucagon, effect on gluconeogenesis, 105, 163
glucokinase (*Dictyostelium*), 320
"glucokinase", mammalian (i.e. hexokinase D), 38-39, 347-348
gluconeogenesis, 63, 161-163
  comparison of theoretical treatments, 101-118
    biochemical systems theory, 113-117
    Crabtree-Newsholme theory, 112-113
    matrix method of Heinrich and Rapoport, 109-111, 116
    metabolic control analysis, 101-109
  control distribution, 107
  reactions of, 102, 103
  oligomycin, effect of, 64, 345
  rotenone, effect of, 65, 344, 346
glucose-lactose interactions in hepatocytes, 347-350
glucose 6-phosphatase, 320
glucose 6-phosphate dehydrogenase, 320
glutamate dehydrogenase, 260, 272-274, 320, 321
glutaminase, 378
glutamine catabolism, 378-379
glutamine synthetase, 183-194, 201
  ammonia, response to, 185
glyceraldehyde 3-phosphate dehydrogenase, 212, 260, 274, 275-277, 321
glycerol phosphate dehydrogenase, 274, 275-277
glycerophosphate dehydrogenase, 260
glycinamide ribonucleotide transformylase, 385-387
glycogenolysis,
  regulation, 209-217
  synthetic particles, 213-214
glycogen phosphorylase, 5, 178, 210-211, 213-215, 320, 399
glycogen synthase, 320
glycolysis, 14, 32, 43, 46-47, 63
  analysis by matrix method, 109
  control distribution
    in erythrocytes, 334-338
    in rat liver, 232, 237
  glucose, control by, 347-348
  oscillations, 303-313
GOLDBETER, A., 173-182
  with Decroly (1982), 298, 310, 312
  with Koshland (1981), 174, 175, 176, 177, 189, 195, 196
  with Koshland (1982), 178, 179, 196
  with Koshland (1984), 180, 195, 196, 200
  with Koshland (1987), 180, 181, 190-191, 196, 209
  with Koshland (THIS SYMPOSIUM), 201 (footnote)
Goldman, D. E., 336
Grazi, E., 275
Greengard, P., 173, 176
GREGORY, R. B., 343-350
GRIVELL, A. R., 343-350
GROEN, A. K., 101-118
  with five others (1982), 105, 161-163, 385
  with four others (1982), 13, 62, 63, 64, 65, 161-163, 236, 328, 343-344, 346, 430
  with van Roermund, Vervoorn and Tager (1986), 109, 159, 161-163
  with Vervoorn, van der Meer and Tager (1983), 63, 105, 108
  with Westerhoff (THIS SYMPOSIUM), 66, 411, 424
Gross, D. J., 37
Große, H., 376
growth, bacterial, 63
Gruppuso, P. A., 210
guanine aminohydrolase, 163-168
Guldberg, 71
GURNACK, M. E., 209-217
Gutfreund, H., 275

haemoglobin, Hill coefficient, 212
Hall, B. G. 393
Hallenbeck, P. C., 209, 214
Hammett, L. P., 46
Hanes, C. S., 261
HANLON, M., 385-387; 386
Hartl, D. L., 390, 396, 397
Haschke, R. H., 209
Hayashi, K., 223
Haystead, T. A., 206
Hearn, A. C., 141
Hearon, 71
Heilmeyer, L. M. G., 209, 214
HEINRICH, R., 329-342
  (1985), 63
  (THIS SYMPOSIUM), 53, 66
  with S. M. Rapoport (1983), 159
  with S. M. Rapoport and T. A. Rapoport (1977), 24, 52, 53, 54, 59, 65, 111, 112, 117, 265, 334, 338, 400, 401
  with T. A. Rapoport (1973), 259
  with T. A. Rapoport (1974), 5, 13, 18, 31, 33,

48, 51-52, 53, 69, 81, 89, 101, 129, 139, 149, 231, 239, 251, 252, 253, 265, 297, 318, 329, 332, 343, 353, 377, 380, 413, 414, 423
with T. A. Rapoport (1975), 59, 63, 89, 109, 149, 282, 301, 332
with T. A. Rapoport (1980), 63
with other authors, 159, 334, 336, 338, 339, 340, 341-342
Heinrichs, M., 309
Heldt, H. W., 365, 366
HENLY, D. C., 343-350
Henri, V., 71
hepatocyte metabolism, 63, 343-350 (*see also* liver parenchymal cells, 102-117)
Hers, H.-G., 32
Herzog, B., 365, 367
HESS, B., 303-313; 35, 47, 264, 271, 281, 298, 302, 304, 305, 307, 310, 311
heterokaryons, 160, 401, 430
hexokinase, 46, 338
hexokinase D ("glucokinase"), 38-39, 347-348
hierarchy of control, 399-405
democratic, 404-405
dictatorial, 401-404
Higgins, C. F., 407
HIGGINS, J., 41-49; 31, 42, 45, 46, 48, 298, 328, 330, 377
high enzyme concentration, 355
Hill, A. V., 12, 34, 71
Hill coefficient, 11, 173, 195, 202
individual proteins, 212
relationship to cooperativity index of Taketa and Pogell, 176, 201, 212
pseudo, 212
Hill equation, 12, 34-35, 100, 285-286
Hirschman, J., 192
histone gene expression, 406-407
HOFMEYR, J.-H. S., 17-26, 239-248, 435
(1986), 141, 143, 144, 245, 246, 435
(1989), 221, 239-240, 241
(THIS SYMPOSIUM), 221, 425-426
with Kacser and van der Merwe (1986), 58, 60, 61, 62, 63, 246
Hohorst, H., 267
Holmes, W. F., 42
Holness, M., 63
Holzer, H., 184, 267
Holzhutter, H.-G., 228
Horvath, C., 291
Huber, J. L., 365
Huber, S. C., 364
Hue, L., 101
Hunter, T., 173, 176
Huntington's Chorea, 381
Huxley, 71
hysteresis, 292-296, 303-313

IBM PC, programs for, 86, 433-436
immune networks, 84
inactivation, detection, 299
independence, 54, 251, 253-254
indexes of cooperativity, relationships between, 176, 201, 212
indoleamine 2,3-dioxygenase, 379-382
inducer, for modulating enzyme activity, 63
induction, 248
infinite kinetic order, 6, 16, 21
inhibitors, use in control analysis, 64-65, 161-162, 343-347, 385-386
interconvertible enzymes, 173-217, 422
activation weaker than inhibition, 180, 203-204
allosteric control, comparison with, 191-192
amplitude, 188
catalytic/specific effects, 197
control-pattern analysis, 248
cooperativity (apparent), 176-177, 188-189, 212
energy cost, 174, 180-181, 190-191
flexibility and integration, 189-190
glutamine synthetase, 183-194
maximum sensitivity, 195-207
multicyclic cascades, 178-180, 192-193, 204
multi-step ultrasensitivity, 179-180
muscle glycogenolysis, 209-217
okadaic acid, effect on muscle, 204-206
propagation of ultrasensitivity, 178-179
rate amplification, 187, 190
saturation of converter enzymes, 175, 176, 202
sensitivity amplification, 179, 188-189
signal amplification, 187-188
terminology, 196
threshold effects, 176
transcriptional regulation and, 192-193
zero-order effects in, 176
interferon γ, effect on tryptophan homeostasis, 380
intermittency, 307
invariants of biochemical systems, 121
ion channels, regulation, 176
IRVINE, D. H., 433-434; 84, 85-86, 89
isocitrate dehydrogenase, 178, 189, 320, 321, 322

Jacobi matrix, 332, 333, 342
Johnson Foundation, 42, 45, 46-48
Jones, T. W. A., 367

KACSER, H., 251-257, 429-432
(1983), 251, 252, 256, 259, 343, 406
(THIS SYMPOSIUM), 427

KACSER, H. (*continued*)
with Burns (1973), 5, 6, 13, 15, 18, 19, 24, 25, 31, 33, 53, 58, 61, 64, 65, 69, 81, 89, 101, 129, 139, 140, 144, 149, 173, 227, 231, 235, 239, 251, 252, 256, 259, 265, 297, 318, 329, 332, 353, 363, 377, 385, 394, 400, 413, 414, 424
with Burns (1979), 162, 223, 251, 256, 259, 265, 343, 344, 380, 403
with Burns (1981), 13, 55, 65, 160, 394
with Middleton (1983), 328
with Porteous (1987), 197, 252, 399
with Sauro (1990), 255
with Sauro and Acerenza (THIS SYMPOSIUM), 54, 145, 254, 255, 302, 349, 355
kappa elasticity coefficient, 61-62, 63
Keener, J., 192-193
KELETI, T., 259-270; 255, 256, 259, 260, 261, 263, 264, 265, 266, 267, 271, 272, 275, 283, 349-350
Kell, D. B., 15, 60, 105, 117, 142, 239, 345, 349, 354, 399
KELLERSHOHN, N., 291-296
Kelley, J. K., 386
Kelly, P. J., 318
Kilberg, M. S., 378
Kimura, M., 389
kinetic mechanisms, independence from control analysis, 256-257
kinetic order, 4, 6-8, 21, 69
equivalent to elasticity, 76
sensitivity, 75
kinetic power, 259-264
King, E. L., 224, 240
Kirchhoff, G., 71
Kirchhoff's laws, 74-75, 76-77, 79
Kirschner, K., 212
Kirschner, M., 406
Klingenberg, M., 267
Knowles, J. R., 263
KNOWLES, R. G., 15-17, 377-384, 385-387; 17, 425
Kodama, I., 204
Kohn, M. C., 298, 303
Kornberg, H. L., 349
KOSHLAND, D. E., 173-182
(1979, 1980, 1981), 291
with Goldbeter (1981, 1982, 1984, 1987, THIS SYMPOSIUM): *see* Goldbeter, A.
with Goldbeter and Stock (1982), 173
with LaPorte (1983), 178, 189
with Némethy and Filmer (1966), 37, 173, 262
with Walsh (1984), 159, 401
Koshland-Némethy-Filmer model, 267 (*see also* sequential model)
KOSTER, J. G., 399-412; 406, 407, 411
Krebs, E. G., 173, 176
Krebs, H. A., 46-47, 317, 325, 349
Krebs cycle, 61 (*see also* citric acid cycle, tricarboxylic acid cycle)
Kruckeberg, A. L., 63, 368
Kubicek, M., 223
Kurganov, B. I., 347
Kustu, S., 192-193
Kvassman, J., 275, 276
kynurenine pathway, 381

LaBaume, L. B., 139, 140
lactose, interactions with glucose in hepatocytes, 347-350
lactose (*lac*) operon, 51
molecular adaptation, 389-398
Lagrange multipliers, 340, 341
LÄMMEL, D., 351-361; 82, 252 (footnote)
Laplace, P., 71
LaPorte, D. C., 159, 178, 189
Larondelle, Y., 366
Larter, R., 303
Lefkowitz, R. J., 176
Leibniz, G. W., 71
LETELLIER, T., 129-138, 433
Liao, J. C., 35
Lightfoot, E. N., 35
Lilley, T. McC., 364
linear formalism, 71
Lineweaver, H., 261
Lipmann, F., 190
liver parenchymal cells, gluconeogenesis, 102-117 (*see* gluconeogenesis for detailed entries; *see also* hepatocyte metabolism, 63, 343-350)
local effects, chain of, 244-246
local properties, 24, 157-159, 226
relationship to global properties, 158, 251, 252
logarithmic gain, 75
equivalent to response coefficient, 76
loops, metabolic, 60-61, 240, 246, 404, 410, 416
Lorenz, E., 303
Low, S., 378
L'vov, V. S., 304
Lyapunov exponent, 302, 305-306, 307

McClure, W. R., 281
MacDonald, F. D., 366, 372-373
McLean, E. W., 386
macroscopic models, 222-224, 274
Magasanik, B., 178, 192
malate, effect on citric acid cycle, 326

malate dehydrogenase, 260, 320, 321, 323, 325
malic enzyme, 318, 320, 321, 323, 324, 325
Malraison, G., 314
Manneville-Pommeau route to chaos, 307
Mantel, M., 184
Marek, M., 223
MARKUS, M., 303-313; 298, 302, 304, 305, 307, 310
Mason, 71
master reaction, principle of, 45-47
Masters, C. J., 259, 278, 347
Mateo, F., 232
matrix methods,
  Fell and Sauro, 139-148
  Heinrich and Rapoport, 109-112
matrix representation of metabolic models, 104, 121-127, 130
Mayr, G. W., 277
MAZAT, J.-P, 11-13, 129-138, 433; 12, 26, 63, 66, 82, 143, 145, 161-162, 422-423
mechanism, independence from control analysis, 256-257
Meijer, A. F., 344
MEINKE, M. H., 209-217; 178, 190, 209, 211
MELÉNDEZ-HEVIA, E., 231-238; 160, 237, 302
membrane transport, 335-336
memory, 291, 295
MENDES, P., 221-224, 434
Menten, M. L., 71, 260
Menzel, R., 407, 411
3-mercaptopicolinic acid, calculation of elasticity of oxaloacetate transport, 163
metabolic control theory (metabolic control analysis), 31, 33, 51-67, 89
  compared with other theories, 101-118, 410-411
  gluconeogenesis, 103-109
  structural aspects, 121-127, 129-138
  variant of biochemical systems theory, 69, 74, 76, 81-83
    aggregation strategy, 97
metabolic-osmotic model, 334-336
metabolic pathway, definition, 231, 232, 236-237, 284
metabolite control coefficient, 60, 381-382 (*see also* control coefficient)
METAMOD (computer program), 435
MetaModel (computer program), 435
METASIM (computer program), 318, 319, 328
meta-steady states, 291-296
Meyer, F., 209, 214
Michaelis, L., 71, 260
Michaelis-Menten equation, 4, 13-14, 41, 92, 260-261, 285
  deviations from, 34, 35, 39
  reversible, 21
Michaelis-Menten formalism, 71-72
microcompartmentation, 271
microscopic models, 222-224, 274
Middleton, R. J., 160, 328
minimum time, as criterion of performance, 150
mitochondrial respiration, 63, 64, 161, 236
Mizraji, E., 298
mnemonical model, 292
model building,
  computer, 6, 12-13, 35, 41, 42-43, 48
  in relation to classical ideas of regulation, 3-11
  macroscopic and microscopic, 222-224
modulated enzyme, 241
modulation of enzyme activity or concentration, 8, 23-24, 63
moiety-conserved cycles, 57, 60, 61, 246-248
molecular adaptation, 389-398
molecular biology, control mechanisms, 51
molecular democracy, 259
molecular evolution, neutral theory, 389
MONIZ BARRETO, J., 221-224
monocyclic cascade, 174, 186
Monod, J., 12, 34-37, 173, 261-262
Monod-Wyman-Changeux model, 34-36, 267 (*see also* symmetry model, 261-262)
Moreno-Sánchez, R., 63
mRNA, translation, 63
MULLIN, R., 385-387
multienzyme complexes, 282, 287
multienzyme systems, 35, 266
multifunctional proteins, 282, 287
multi-step ultrasensitivity, 179-180
Mura, U., 185, 188
Murata, M., 204
muscle,
  contraction, 209
  effect of okadaic acid, 204-206
  glycogenolysis, 209-217
mutants, use for varying enzyme activity, 63, 160, 367-368, 401, 430
myosin light-chain phosphorylation, 204-206

NAD^+/NADH and $NADP^+$/NADPH ratios, 21, 52
NADH dehydrogenase, 344, 346
Na/K ATPase, 338
Naparstek, A., 173
natural selection,
  control and, 395-397
  in relation to neutral theory, 389
Navier-Stokes equations, 303

near-equilibrium enzymes, control of metabolism and, 107, 267, 377-378
networks
development of understanding, 71
theory, 72
Needham, J., 71
Neet, K. E., 292
Nestler, E. J., 173, 176
Neuhaus, H. E., 368
Neumann, E., 291
Neurospora,
arginine synthesis, 63
arom complex, 282-283
heterokaryons, 160, 401, 430
neutral theory of molecular evolution, 389
Newport, J., 406
Newsholme, E. A., 5, 16, 18, 20, 33, 40, 69, 78-79, 89, 101, 112-113, 144, 149, 227, 266, 424
Newton, I., 44, 71
Nicholson, S., 287
Niemeyer, H., 39, 196
Nikaido, H., 392, 396
Ninfa, A. J., 192
nomenclature, 112
non-additivity, 253-256
non-independence, 253-256
nonlinearity, responsible for interesting properties of the cell, 70
Northrop, D. B., 261
norvaline, use in study of citrulline biosynthesis, 162

Ochs, R., S., 378
Ockham, William of, 33, 327
okadaic acid, 204-206
Okuno, E., 381
oligomycin, as inhibitor of hepatocyte metabolism, 344-346
one-encounter-type metabolite transfer, 271
operon, 51, 84, 389-398
optimal stability, 303-313
optimal track, as criterion of performance, 150
order, kinetic, 4, 6-8, 21, 69
equivalent to elasticity, 76
sensitivity, 75
Origin of Species, the (C. Darwin), 4
originality, nature of, 36-38
ornithine transcarbamoylase, elasticity for carbamoyl phosphate, 163
Orosz, F., 260, 273, 275, 276, 277
oscillatory reactions, 48, 53, 298, 299, 302, 303-313
osmotic model of erythrocyte metabolism, 334-338
OVÁDI, J., 271-279; 255, 256, 257, 259, 260, 267, 271, 272, 273, 275, 276, 277, 288
oxidative phosphorylation, 13
channelling, 345-346
effect of oligomycin, 65, 344-346
effect of rotenone, 65, 345-346
2-oxoglutarate dehydrogenase, 320, 321

Pachot, P., 298
Packard, N. H., 304, 305
parameter (π) elasticities, 252-256
Pardee, A. B., 422, 423
Parry, M. J., 38
partially internal regulators, 113
partitioned regulatory coefficient, 227-230
pathway, definition of, 231, 232, 236-237, 284
Patthy, L., 260, 275
Penny, D., 33
pentose phosphate pathway, 334
performance indices, 149-156, 340
period-doubling cascades, 307
permease (lactose), 392-397
peroxidase, 41, 42
perturbation analysis, 307-309
Pettersson, G., 360
Pettit, F. H., 201
phase space dimension, 303, 304
phenylalanine hydroxylase, 377
philosophy of science, 13, 32
PHILLIPS, J. W., 343-350
phosphate (inorganic), and sensitivity catastrophe in cytosol, 359
phosphoenolpyruvate carboxykinase, 344
phosphofructokinase (6-phosphofructo-1-kinase), 47, 260, 274, 277-279, 320, 338
6-phosphofructo-2-kinase, 189 (*see also* fructose 6-phosphate 2-kinase, 365, 366)
6-phosphogluconate dehydrogenase, 320
phosphoglucose isomerase, 320, 363, 367-371
phosphoglycerate kinase, 260
2-phosphoglycerate mutase, 260
phosphoribulokinase, 358-359
phosphoribosylpyrophosphate amidotransferase, 385
phosphorylase, 5, 178, 210-211, 213-215, 320, 399
phosphorylase *a-b* cycle, 209
phosphorylase *b*, 190
photosynthesis, 63,
light-driven reactions, 354-355
sucrose synthesis, 363-376
π elasticities, 252-256
Pickett-Gies, C. A., 214
plasmids, for modulating enzyme activity, 63, 401
Pogell, B. M., 176, 197

POGSON, C. I., 377-384, 385-387; 379, 381
polyethylene glycol, promoter of complex formation, 276
PONCES FREIRE, A., 221-224
Popper, K., 13, 32-33, 423, 424
porins, 392, 396
PORTEOUS, J. W., 51-67; 15, 33, 62, 81-82, 103, 197, 225, 252, 399
power-law formalism, 69-87, 89
  compared with other formalisms, 71-72
  roots of, 71
Preiss, J., 366
Press, W. H., 85
principles, general, none in biology, 4
priority in discovery, 38-39, 252 (footnote)
programs (computer), 85-86, 138, 141, 145, 318, 433-436
protein/DNA ratio in development, 406-407
protein kinases and phosphatases, 174, 180, 189, 190, 204-206, 209-217
proton leak, and control of respiration, 64
protonophore, effect on DNA supercoiling, 409
Pryor, H. J., 63, 343
purine biosynthesis, 63, 385-387
purine catabolism, 163-168
pyruvate dehydrogenase, 320, 321
  complex, 201
pyruvate kinase,
  elasticity for phosphoenolpyruvate, 163
  gluconeogenesis, 103, 107-109
  glycolytic oscillations and, 306, 307

quasiperiodicity, 307, 308
Quick, P., 364, 366
quinolinate metabolism, 381-382
quinolinate phosphoribosyltransferase, 381

Rabitz, H. K., 149, 150, 303
radioactive label, use to measure channelling, 274-275
Rameaux, 71
Rapoport, S. M., 59, 159, 259
Rapoport, T. A.,
  with Heinrich (1973, 1974, 1975, 1976): *see* Heinrich, R.
  with Heinrich, Jacobasch and S. M. Rapoport (1974), 63, 159, 334, 338
  with Heinrich (1975), 53, 63, 89, 109, 149, 282, 301, 332
  with Heinrich and S. M. Rapoport (1976), 109, 159, 334, 338
rate-constant sensitivity, 75
receptors, regulation, 176
recessivity, 13
REDER, C., 121-127, 129-138
  (1986), 125, 129, 135, 138, 141, 332
  (1988), 121, 125, 126, 129, 135, 138, 141, 143, 144, 252, 256, 332, 352, 353, 433
  (THIS SYMPOSIUM), 66, 116
  with Mazat (1988), 126, 143, 433
  with Mazat and Letellier (THIS SYMPOSIUM), 66
REDUCE (computer program), 141
reductionism, 70
Rees, D. C., 436
reference flux, 241
reference metabolite, 241
reflection coefficients, 41-45
refrigerator, analogy to metabolic system, 417-418, 431-432
regulability, 15-16, 17, 382-383
regulation,
  classical view of, 3-11, 15, 17
  performance indices, 155
  relationship to control, 21-23, 225-230, 382-383, 409-410, 417
regulatory coefficient, partitioned, 227-230
regulatory importance, 15-16, 17, 21, 382-383, 425
Reich, J. G., 267, 340
relaxation times of erythrocyte glycolysis, 341
repression, 248, 404
respiration, 63, 64, 161, 236, 344, 345, 346
response coefficient, 15, 24, 62, 158, 197, 225, 227, 329-330
  equivalent to logarithmic gain, 76
  interconvertible enzyme systems, 199
  use as term for sensitivity index, 197
reticulocytes, translation in, 63
Revzin, A., 291
RHEE, S. G., 183-194; 178, 183, 184, 185, 186, 189, 196, 201
ribulose 1,5-bisphosphate carboxylase, 355-357, 360
RICARD, J., 291-296; 33, 291, 292, 312
Rich, V., 34
RNA metabolism, 402-404
RNA, translation, 63
Robinson, S. P., 359
rotenone, use for control analysis, 65, 344, 346
Roughton, F. J. W., 42
RUDD, K. E., 399-412
Ruelle-Takens route to chaos, 307
Runge-Kutta method, 223
Ryde-Pettersson, U., 360

$S_{0.5}$, regulation by, 7, 10-11, 23, 26

Sakamoto, N., 223
Salerno, C., 260, 273
SALTER, M., 15-17, 377-384, 385-387; 16, 17, 63, 160, 161, 235, 377, 425, 430
Sanders, D., 367
Sarrus, 71
saturation, 4, 5, 20
  converter enzymes in interconvertible systems, 176, 202
SAURO, H. M., 13-15, 139-148, 225-230, 251-257
  (1986), 301
  (1987), 159 (footnote)
  (THIS SYMPOSIUM), 257, 431
  with Fell (1985), 58, 60, 82, 104, 129, 138, 139, 141, 143, 144, 149, 158, 221, 239, 252, 256, 354
  with Fell (1987), 5-9, 10, 14-15, 25-26, 117, 141, 158, 221, 239, 255, 256, 416
  with Fell (THIS SYMPOSIUM), 39, 423-425
  with Fell and Small (THIS SYMPOSIUM), 221
  with Kacser (1990), 255
  with Kacser and Acerenza (THIS SYMPOSIUM), 145, 302, 349, 355
  with Small and Fell (1987), 60, 104, 138, 141, 142, 143
SAVAGEAU, M. A., 69-87, 433-434
  (1969), 31, 33, 69, 72, 73, 75, 83, 89, 91, 101, 149, 239
  (1970), 31, 33, 72, 75, 89
  (1971), 72, 73, 82, 89
  (1972), 72, 73, 84, 89, 259, 91
  (1975), 91
  (1976), 18, 31, 33, 66, 84, 91, 113, 117, 149, 239, 252, 259, 343, 410
  (1979), 91
  (1987), 149
  (1989), 84
  (THIS SYMPOSIUM), 33, 111, 113, 145, 239, 413, 415
  with Irvine (1985), 84
  with Irvine (1990), 85-86
  with Sorribas (1989), 69, 73, 74, 76, 78, 79, 82, 83, 85, 89, 91, 93, 97, 98, 99
  with Voit (1987), 73, 83, 84, 85, 91, 95, 96
  with Voit and Irvine (1987), 82, 89, 144, 145-147
SCAMP (computer program), 301
scanning tunnelling microscopy, 210, 214-215, 217
Schauer, M., 339
Schneider, F. W., 291, 312
Schuster, H. G., 303
Schuster, R., 339
Schuster, S., 334, 339, 342
Segal, A., 187, 201
Selkov, E. E., 334
Selwyn, M. J., 299
Senior, P., 185
sensing potential, 294
sensitivity index, 197
sequence divergence, constant rates of, 389
sequential model (of cooperativity), 262
serine biosynthesis, illustration of matrix method, 139-141
Shacter, E., 176, 181, 189, 190, 209
shell-fish poisoning, 204
Shibata, S., 204
shikimate kinase, 282-283
shortening of metabolic pathways, 231-238
sigmoid rate-concentration curve, 7
signals
  amplification, 187-188
  sensing by enzymes, 291-296
simplicity, meaning of, 12
simulation of pathway behaviour, 165, 221-224, 284
Slater, E. C., 37-38
SMALL, J. R., 139-148; 60, 142, 158, 195, 196, 221, 255
SMITH, G. K., 385-387; 63, 386
Snell, K., 139, 140, 159
socialism, supramolecular, 259
Société Française de Biologie Théorique, 452
sodium-potassium ATPase, 338
Solti, M., 260
Sonntag, I., 341
Sorribas, A., 60, 69, 74, 76, 79, 82, 83, 85, 89, 91, 97, 98, 99, 159
SOULIÉ, J.-M., 291-296; 291
specific/catalytic effects in interconvertible enzyme systems, 197, 198-202
specificity constant, 261
Spencer, H., 71
spliceosome, 402
spreadsheet, electronic, metabolic modelling with, 435-436
Srere, P. A., 209, 255, 259, 271, 278
Srivastava, D. K., 259, 271, 275
S-system representation, 69-87, 91
  accuracy, 91, 92-97
  applications, 84, 113-116
  detailed example, 74-76
  compared with other representations, 81
stability,
  dynamic, 19
  structural, 19
  optimal, 303-313

STADTMAN, E. R., 183-194; 174, 178-179, 183, 184, 185, 187, 188, 189, 190, 195, 196, 201, 422
starch synthesis, 363-376
steady state,
  geometrical representation, 130-131
  perturbation, 131-133
steep transitions, 173
STEFFEN, K., 351-361; 82, 252 (footnote)
STEINHORN, D. M., 209-217
stiff differential equations, methods for, 223
STITT, M., 363-376; 63, 363, 364, 365, 366, 430
stoicheiometry matrix, 143, 355-357
Stone, T. W., 381
Storer, A. C., 38, 39
structural approach to metabolic analysis,
  geometrical aspects, 129-138
  theoretical aspects, 121-127
Stuart, F., 160
substrate concentration as regulator, 7-8
substrate cycling, 107, 142
subunit association-dissociation, 248
succinate dehydrogenase, 320, 321, 322, 323
sucrose phosphatase, 366
sucrose phosphate synthase, 364-365
sucrose synthesis, photosynthetic, 363-376
Suganuma, M., 204
summation property, 13, 48, 58-60, 81-82, 103, 126, 140, 251-252, 331, 332, 394, 408-409, 430
  apparent failure in *Dictyostelium* simulation, 318, 323, 324-325, 327, 328
  apparent failure in hepatocyte metabolism, 65, 344
  channelling, effect of, 349-350
  geometrical interpretation, 137
  hierarchy of control and, 400
  non-additivity and non-independence, 254-256
supercoiling of DNA, 407-409
supramolecular socialism, 259
symbolism, 103
symmetry model (of cooperativity), 261-262 (*see also* Monod-Wyman-Changeux model, 34-36, 267)
system representations, 73-74
Szabolcsi, G., 260

Tachibana, Y., 204
Tager, J. M., 63, 102, 162, 343
Takai, A., 204-206
Taketa, K., 176, 197
Taylor series representation of functional relationships, 72-73, 91
Teissier, 71
temporal analysis, 281-290, 297-302 (*see also* time)
terminal control, as criterion of performance, 150
terminology, 103
tetrarhythmicity, 310
thermodynamic control, 347-350
Theorell, H. , 42
theorems of control analysis, 353, 403, 405
theory, nature of in metabolic control, 31-40
Thomas, G. B., 72
thought experiments, 54
threshold, 173, 178, 365-366
Tikhonov theorem, 338
time,
  invariants, 299-300, 303
  transient (*or* transition), 38, 223, 264, 267, 271-275, 281-290, 297-302
time-dependent control coefficients, 333-334
time hierarchy, 341-342
time-limiting enzyme, 282
titration of enzymes, 160, 231-238
Tomovic, R., 149, 150
Tompa, P., 260, 271, 272
topoisomerase, 407-408
TORRES, N. V., 231-238; 63, 159, 160, 232, 235, 237, 301, 328, 430
totally external regulator, 113
transient (*or* transition) time, 38, 223, 264, 267, 271-275, 281-290
transport, 335-336, 378-379
trehalose 6-phosphate synthase, 320
Trempe, M. R., 214
Trends in Biochemical Sciences, vii, 333
tricarboxylic acid cycle, 317 (*see also* citric acid cycle, Krebs cycle)
triose phosphate isomerase, 260, 274
trirhythmicity, 310
Trombetta, G., 275
tryptophan 2,3-dioxygenase, 161, 377-382, 383
tryptophan homeostasis, 379-381
Tse-Dinh, Y.-C., 407
Tsong, T. Y., 349
tunnelling electron microscopy, 210, 214-215, 217
two-substrate reactions, kinetic power and, 262-264
tyrosine aminotransferase, 377

UDPglucose pyrophosphorylase, 320
ultrasensitivity, zero-order, 173-182, 210-212
Umbarger, H. E., 71, 422, 423
uncompetitive/competitive effects in interconvertible enzyme systems: *see* catalytic/specific effects
uncompetitive inhibition in metabolic pathways, 199-200

uniqueness of coefficients, 353-354
unpredictability, 303-313
upstream effects, 227-230
uric acid biosynthesis, 163-168
uridine phosphorylase, 320
uridylation/deuridylation cycle, 183-194

Vaara, M., 392, 396
Van Dam, K., 63, 66, 110, 399, 400, 411
Van der Meer, R., 102
Van Schaftingen, E., 32, 189
VAN WORKUM, M., 399-412
Vas, M., 260, 275
VAZ GOMES, A., 221-224
Veech, R. L., 267
Vértessy, B., 260, 264, 265, 266, 267, 275
V_{max}
  as regulator, 8, 11, 23
  in vivo, 320
VOIT, E. O., 89-100, 433-434
  (THIS SYMPOSIUM), 33, 83, 85, 111, 113, 145-147, 239
  with Irvine and Savageau (1989), 75, 86
  with Savageau (1987), 73, 83, 84, 85, 91, 92, 95, 96, 100, 147
volume, cellular, control coefficients, 337-338
Vukobratovic, M., 149, 150

Waage, 71
Waley, S. G., 340
Walker, D. G., 38
WALLACE, P. G., 343-350
Wallach, J., 98
Walsh, D. A., 209, 214
Walsh, K., 159, 401
Wanders, R. J. A., 60, 63, 161-163
Wegscheider condition, 339
Weiner, H., 364
WELCH, G. R., 343-350; 259, 261, 263, 264, 265, 266, 267, 271, 282, 287, 347, 349-350
Werner, A., 159, 334, 336
WESTERHOFF, H. V., 101-118, 399-412
  (1985), 63
  (1989), 399
  with Chen (1984), 59, 129, 144, 149, 251, 332, 353, 400, 405
  with five others (1987), 63
  with Groen (THIS SYMPOSIUM), 66, 411, 424
  with Groen and Wanders (1984), 114, 173, 266
  with Kell (1986), 239
  with Kell (1987), 15, 60, 104, 117, 142, 354
  with Kell (1988), 349
  with Koster, Van Workum and Rudd (THIS SYMPOSIUM), 63
  with Van Dam (1987), 63, 110, 399, 400
Wigge, B., 364
Wilson, A. C., 389
Wilson, D. M, 396
Wilson, T. H., 393
Winkler, H. H., 393
Wirtz, W., 364
Wistow, G. J., 321
Woese, C. R., 389
Wolf, A., 305
Wong, S. C., 355
Woodland, H., 407
Woodrow, I. E., 63, 360
WRIGHT, B.E., 317-328; 48, 318
Wright, J. K., 393
Wu, H.-Y., 408, 411
Wurster, B., 264, 271

xanthine oxidase, 163-168
Xenopus laevis, development, 406-407

Yang, C. N., 37
YANG, R., 209-217
YANG, X., 209-217
Yates, R. A., 422, 423

zero-order kinetics, 5, 6
zero-order ultrasensitivity, 173-182, 202, 210-212