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_Tolga Bilgen_

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I thank the authors of this book who are, except for my wife, all former graduate students or postdoctoral fellows from my lab. We are a "serial scientific lab family" in that people come and then go, moving on to their new scientific futures over time. Each new arrival first learns from the lab, then enriches the lab with their ideas and hard work, and then moves on leaving a luminous beacon in the form of scientific achievements that allow the next students to find their way to the edge of knowledge and then plunge into the unknown. I have always operated as a "time vampire" — taking years from the young, asking long hours as well as physical and mental effort — and returning to them the immortality of adding to the totality of scientific knowledge. The authors of this book are only a few of the many students and fellows who have passed through my lab and who have succeeded at science and built productive careers. I am by far the big winner in this bargain of time for opportunity and I truly appreciate the chance the Cosmos has bequeathed me in allowing me to hold onto their coattails for a short time while they learned and applied the scientific method.

I must also thank the mentors who I have had throughout my career — there are several who have been key at different forks in the road but two who must be named. Shining above the rest are my father, Dr. Arthur G. Storey, who instilled in me a desperate work ethic and a need for accomplishment (regardless of the arena), and Dr. Peter W. Hochachka who gave direction and substance to my desire to succeed and introduced me to the elegance of minimalist experimentation and the power of "Synthetic Intuition." Two great fathers in one lifetime are more than I deserve.

In producing this book, I would also like to thank Luna Han, my editor at Wiley, for her support and encouragement, and Christine Punzo for keeping us on track during production and proofreading. Big thanks also to my wife, Jan, for her scintillating writing, and her "eagle eye" and attention to detail during the writing and editing process.

Ken Storey
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Key Concepts

The ability to control the rates of metabolic processes in response to changes in the internal or external environment is an indispensable attribute of living cells that must have arisen with life’s origin. This adaptability is necessary for conserving the stability of the intracellular environment (homeostasis), which is, in turn, essential for maintaining an efficient functional state. In the absence of such control, all metabolic processes would achieve a state of equilibrium with the external environment. For example, the intracellular storage of a fuel macromolecule such as glycogen would be impossible since there is an enzyme (glycogen phosphorylase) dedicated to catalyzing the breakdown of this storage polyglucan into its constituent glucosyl units. Obviously, the existence of glycogen as an important energy store in animal tissues implies that the activity of glycogen phosphorylase is carefully controlled so as to allow this fuel to be utilized as dictated by the needs of the organism. Indeed, elaborate regulatory mechanisms have been discovered that affect glycogen phosphorylase activity, thereby allowing wide variations in the rate of glycogen breakdown in vivo. The aim of this chapter is to outline the biochemical regulatory mechanisms that are believed to be the most important in metabolic control. Practical aspects for the study of metabolism and its control, as well as the advantages and disadvantages of qualitative versus quantitative approaches to metabolic control, will also be highlighted.

Metabolic Renaissance in Postgenome Era?

The remarkable advances in molecular genetics that have occurred over the past couple of decades have somewhat eclipsed areas of traditional biochemistry such as protein chemistry, enzymology, and metabolic control. With many genomes sequenced and others nearing completion, the next step is the less straightforward task of analyzing the expression and function of gene products (proteins), as well as more thoroughly elucidating metabolism and its control. The task of completing the picture of all cellular proteins, their actions and reactions, is one of the biggest challenges facing life science researchers today. Although molecular biology has generated many impressive techniques [e.g., protein overexpression, site-directed mutagenesis, metabolic engineering, complementary deoxyribonucleic acid (cDNA) microarrays, etc.] for assessing various aspects of protein/enzyme structure–function and regulatory control, one cannot deduce the properties of a functional protein or the kinetic and regulatory properties of an enzyme solely from genetic information. Moreover, recent animal, plant, and microbial genome sequencing projects have revealed a plethora of gene sequences that encode proteins having unknown functions. Furthermore, many organisms whose genomes have been sequenced have not had their metabolism extensively studied. Where feasible, their metabolic phenotype is determined using annotated genome sequence data. Thus, there appears to be a resurgence of interest in protein, enzymological, and metabolic research for understanding biological processes in the postgenome era. Efficient approaches are needed for determining: (a) the function of unknown gene products, (b) protein expression in different cells under various conditions, (c) covalent modifications of proteins in response to different stimuli, (d) protein–protein interactions, (d) the relationship between protein structure and protein function, and (e) the sophisticated mechanisms that serve to control the flux of metabolites through specific
metabolic pathways in vivo. Novel methods are also being developed to map proteomes (i.e., the proteins encoded by the genome) and to discover new enzymes of interest.

**Metabolic Engineering**

Since it is now possible to manipulate nucleic acids and gene expression at will, an important goal of biotechnology is to modify (usually enhance) the output of specific biosynthetic pathways via the process of metabolic engineering. Contemporary genetic engineering techniques have created the potential to directly modify the metabolism of a target organism in a desired fashion. However, the ability to manipulate an organism’s genetics has thus far transcended our ability to predict the effects of these manipulations on metabolism. Our lack of a thorough appreciation for, and understanding of, metabolic control is reflected by the meager results from most attempts to apply the powerful tools of molecular biology and genetic transformation to the practical goal of metabolic engineering. Many unsuccessful efforts at so-called shotgun metabolic engineering have been based upon the misguided assumption that if the expression of a gene encoding a particular enzyme is suitably manipulated, then there will be a corresponding change in both the in vivo activity of the encoded enzyme as well as the flux (or rate of movement) of metabolites through the pathway in which the enzyme functions. “Rational” metabolic engineering is a longer-term, but arguably more scientific and interesting process that involves the targeted and purposeful alteration of a specific metabolic pathway. It does not necessarily depend upon altering the concentration of an enzyme but could be carried out by introducing a mutant (or homologous) enzyme with altered control properties. Apart from an ability to manipulate nucleic acids, rational metabolic engineering also requires a strong background in protein/enzyme and metabolic biochemistry.

**Metabolic Regulation versus Metabolic Control**

Although biochemists frequently employ the terms regulation and control interchangeably, the need to discriminate between these terms has been emphasized (Fell, 1997). Metabolic control refers to adjusting the output of a metabolic pathway in response to an external signal. By contrast, metabolic regulation occurs when an organism maintains some variable relatively constant over time, despite fluctuations in external conditions. Homeostasis is therefore a consequence of metabolic regulation, which itself may be a result of metabolic control. For example, the regulation of mammalian blood glucose is largely due to the secreted peptide hormones glucagon (“starved” signal) and insulin (“fed” signal) controlling intracellular metabolism within the liver. In this case, the concentration of blood glucose is regulated (kept constant) mainly by controlling (varying) fluxes of metabolic pathways (i.e., glycogen breakdown versus synthesis, glycolysis, gluconeogenesis) in hepatocytes. Regulation and control are properties of highly elaborate metabolic systems. An ongoing challenge is to link our knowledge of molecular, reductionist-based, enzyme control mechanisms to organism-level explanations of metabolic regulation.

**Complexity of Metabolism and Concept of Biochemical Unity**

As protein catalysts, enzymes vastly accelerate the rates of chemical reactions without themselves undergoing a permanent change. As each cellular reaction is catalyzed by its own enzyme, every cell contains a large number of different enzymes. Although a “simple” prokaryote, such as *Escherichia coli*, is only about 1/500th the size of a typical eukaryotic cell, each *E. coli* cell contains about 3000 different proteins, at least 90% of which are enzymes. The metabolic complexity of all cells is reflected by the many separate enzymatic reactions that make up the metabolic pathways that collectively constitute metabolism.

Despite its complexity, a general understanding of metabolism has been achieved because common solutions to the problem of biochemical design have been evolved. Thus, the types of substrates, cosubstrates, cofactors, fuels, and types of metabolic pathways used are common to most cells. This is the concept of biochemical unity. In general, biochemical unity also applies to metabolic regulation and metabolic control. Comparative biochemistry has revealed that the types of control mechanisms found in metabolic pathways are similar from one organism to the next. However, it is the implementation of these designs—the regulatory details—that cannot only differ widely from species to species but can differ widely for similar metabolic pathways in different cell types of a single organism, or even within different organelles of a single cell.

For example, citrate synthase, which catalyzes the reaction acetyl-CoA + oxaloacetate → citrate + CoA, is controlled in dissimilar manners in different cells. In respiring animal cells, a major function for this enzyme is in the citric acid cycle that operates in the mitochondria in conjunction with oxidative phosphorylation to produce adenosine 5'-triphosphate (ATP). Here, the overall end product, ATP, feeds back to inhibit citrate synthase. This regulatory mechanism is logical since at high ATP levels, the ATP-generating citric acid cycle will then be inhibited, but if ATP levels fall, substrate catabolism by the cycle will speed up. In *E. coli*, however, citrate synthase and the citric acid cycle have a different function. This bacterium lives a mainly anaerobic life, generating its ATP primarily
via the fermentation of glucose by glycolysis. The main role of the citric acid cycle in E. coli is in the generation of biosynthetic precursors and reducing power [reduced nicotinamide adenine dinucleotide (NADH)]. E. coli citrate synthase is unaffected by ATP, but it is inhibited by one of the ultimate end products of the cycle, NADH. Germinating seeds contain a third type of citrate synthase, localized in the glyoxysome. This isozyme is insensitive to both ATP and NADH, and here the enzyme functions as part of the glyoxylate cycle, an indispensable component of the metabolic conversion of fatty acids derived from storage triacylglycerides into sucrose. Thus, the concept of biochemical unity tends to break down when individual metabolic controls are compared. Although the structure and products of a metabolic enzyme or a metabolic pathway may be identical in various organisms, the environment and function of that enzyme or pathway may not be the same. Nevertheless, all metabolic controls have a common basis, and certain regulatory strategies are ubiquitous.

**BASIS OF METABOLIC CONTROL**

**Pacemaker Enzymes**

It is self-evident that the flux of metabolites through any pathway must be closely coordinated with the needs of the cell, tissue, or organism for the final end product(s) of the pathway. The traditional view of metabolic control is that such regulation is accomplished by altering the activity of at least one pacemaker enzyme (or rate-determining step) of the pathway. Thus, a major focus of enzymology has been to characterize these key enzyme reactions—the pacemakers—that are believed to be most important in controlling pathway flux. Substantial efforts have been directed to identifying the pacemaker enzyme(s) of metabolic pathways, as well as the complex mechanisms that serve to modulate the activities of these key enzymes.

**Identification of a Pacemaker Enzyme**

Normally, the pacemaker enzyme(s) of a pathway has a low activity overall, is subject to control by metabolites other than its substrates, and is often positioned as the first committed step of a pathway, directly after major branch points, or at the last step of a “multi-input” pathway. However, such circumstantial evidence for a putative pacemaker enzyme-catalyzed reaction still requires confirmation by techniques that rely upon accurate quantification of the *in vivo* concentrations of the enzyme’s substrate(s) and product(s).

The standard method for determining metabolite concentrations begins with ultrafast freezing of cells/tissues in liquid nitrogen at −196°C. This effectively quenches any reactions that could lead to artifactual alterations in metabolite levels. The frozen tissue is then extracted by homogenization at low pH in cold perchloric acid, which inactivates enzymes that would affect the metabolites of interest. Following centrifugation to remove cell debris and precipitated proteins, the supernatant is neutralized and analyzed for metabolites by appropriate enzymatic or chemical methods. More recently, $^{31}$P nuclear magnetic resonance ($^{31}$P-NMR) has become widely used for determining the intracellular concentrations of phosphate-containing metabolites such as the hexose-phosphates, adenylates, phosphocreatine, and inorganic phosphate. It is now feasible to measure the concentrations of phosphate-containing metabolites in a tissue, perfused organ, or even an intact living organism inserted into the wide-bore magnet of a NMR spectrometer. Results obtained from $^{31}$P-NMR are generally consistent with those obtained by acid extraction techniques. However, noninvasive NMR techniques continue to hold great promise for providing a detailed insight into metabolite levels of living cells, and how these levels may vary following perturbations such as oxygen deprivation, or the addition of metabolites or hormones. Whether obtained via classical or NMR methods, the amount of a metabolite (determined as micromoles per gram of tissue), can also be expressed as concentration [i.e., in millimolars (mM)] in the intracellular water if the water content of the tissue is known.

Accurate determinations of metabolite levels can be difficult, particularly if the same metabolite is distributed between several intracellular compartments. Nevertheless, the availability of metabolite data is quite relevant to determining how and where flux control of a specific metabolic pathway is exerted, that is, probable pacemaker enzyme(s). A major controlling enzyme should theoretically catalyze the slowest step in the pathway. Thus, a reaction that is far from equilibrium *in vivo* is likely to be catalyzed by a pacemaker enzyme (although under certain conditions, enzymes that catalyze reactions close to equilibrium may also be regulatory). The reason that equilibrium is not achieved is that owing to the action of various “fine” metabolic controls (such as feedback allosteric inhibition; see below) there is insufficient active enzyme present to bring the reaction to equilibrium *in vivo*. Hence, a metabolic biochemist interested in identifying important sites of pathway control initially searches for those enzymes that catalyze nonequilibrium reactions *in vivo* (i.e., catalyze irreversible reactions that have a highly negative overall free energy change). This requires measurement of the intracellular concentrations of the particular metabolites involved. Results obtained with a rat heart perfused with glucose are listed in Table 1.1.

For the glycolytic reaction catalyzed by 6-phosphofructokinase (PFK): F6P + ATP → FBP + ADP, the ratio of its intracellular concentrations of products:substrates, known
TABLE 1.1 Intracellular Concentration of Several Metabolites Obtained with a Rat Heart Perfused with Glucose

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Intracellular Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Fructose-6-phosphate (F6P)</td>
<td>0.09</td>
</tr>
<tr>
<td>d-Fructose-1,6-bisphosphate (FBP)</td>
<td>0.02</td>
</tr>
<tr>
<td>ATP</td>
<td>11.5</td>
</tr>
<tr>
<td>ADP</td>
<td>1.3</td>
</tr>
<tr>
<td>AMP</td>
<td>0.17</td>
</tr>
</tbody>
</table>


as the mass action ratio ($\Gamma$), is given by

$$\Gamma = \frac{[\text{FBP}][\text{ADP}]}{[\text{F6P}][\text{ATP}]} = 0.025$$

where F6P is fructose-6-phosphate, FBP is fructose-1,6-bisphosphate and ADP is adenosine 5'-diphosphate. The equilibrium constant ($K_{eq}$) for a chemical reaction, a value that is independent of the presence or absence of a catalyst (enzyme), can be determined in separate experiments under physiological pH, temperature, and pressure. The $K_{eq}$ for the aforementioned reaction is about 1200. Since this value is 48,000-fold greater than the reaction's corresponding $\Gamma$ value in perfused rat heart, it is evident that the reaction in vivo is very far removed from equilibrium and, thus, is essentially irreversible. Therefore, PFK is regarded as a probable pacemaker enzyme of the glycolytic pathway. This is logical because PFK catalyzes the first committed step of glycolysis, that is, the first step that does not form part of other metabolic processes as well. By contrast, for the reaction catalyzed by adenylate kinase: $\text{ATP} + \text{AMP} \leftrightarrow 2\text{ADP}$,

$$\Gamma = \frac{[\text{ADP}^2]}{[\text{ATP}][\text{AMP}]} = 0.85$$

where AMP is adenosine 5'-monophosphate. This is very close to the reaction's $K_{eq}$ value of 0.44. This indicates that rat heart adenylate kinase is sufficiently active in vivo to allow the reaction to remain very close to equilibrium (or readily reversible).

Another method used to identify pacemaker enzyme(s) that has been employed is the "crossover theorem," which states that when pathway flux is enhanced, the substrate concentration of the pacemaker enzyme will decrease and its product concentration will increase (and vice versa for a flux decrease). A crossover in relative metabolite levels between two physiological states indicates where a regulatory signal has acted on a pacemaker enzyme to alter flux. For example, in yeast, as well as in many animal and plant tissues, oxygen deprivation greatly enhances glycolytic flux (owing to the need to generate ATP via anaerobic fermentation). This is associated with an immediate reduction in intracellular [F6P] and an increase in [FBP]. This results in a "positive crossover" between F6P and FBP and indicates that the perturbation (anoxia) has activated PFK in vivo so that the concentration of its substrate, F6P, is lowered whereas that of its product, FBP, is increased. An elevated [FBP] would directly cause an increased flux through the next enzyme in the glycolytic pathway, FBP aldolase (which catalyzes a reaction close to equilibrium and shows no crossover following the aerobic to anaerobic transition). This flux increase would then be transmitted to the remainder of the glycolytic sequence. Crossover analysis clearly identifies PFK as a pacemaker enzyme whose activity can respond appropriately to facilitate the marked stimulation of glycolysis that accompanies the imposition of anoxia stress. This example also underscores an important principle in metabolic biochemistry research: namely, that the full elucidation of pathway control is often dependent on a thorough comparative analysis of a control versus perturbed (i.e., stressed) tissue. Correlation of alterations in metabolic fluxes and metabolite levels that ensue from a perturbation of cells/tissues generates indispensable information for the metabolic biochemist seeking to identify key sites of control in a particular pathway.

Enzyme Purification

From the various approaches described above it is possible to pinpoint the most important sites of control of a metabolic pathway. The next step is to examine the molecular and kinetic properties of the putative pacemaker enzyme(s), particularly those properties that might be involved in the control of pathway flux. Of particular interest are the following questions:

1. What is the enzyme's subunit structure? Pacemaker enzymes are invariably multimeric; that is, in their native state they consist of two or more subunits held together by noncovalent bonds. A more complex protein structure appears to be a prerequisite for a more complex protein function/regulation (i.e., allosterism, etc.).

2. How does the activity of the enzyme vary with alterations in assay pH and substrate(s) concentration(s)? Are hyperbolic (Michaelis–Menten) or sigmoidal (cooperative) substrate saturation kinetics observed? As discussed below, the sigmoidal substrate saturation plot observed for some pacemaker enzymes implies that over a certain range of substrate concentrations, the activity is more sensitive to...
Is the activity of the enzyme controlled by any metabolites (referred to as effectors) that are structurally distinct from its own substrate(s) or product(s)? If so, (a) what is the nature of the interaction (i.e., activation or inhibition) and (b) do these metabolite effectors significantly alter enzyme activity in vitro at concentrations that are known to exist in vivo?

4. Is the activity of the enzyme subject to control by covalent modification such as by reversible protein phosphorylation, and if so, what mechanisms serve to control the activities of the modifying enzymes that catalyze these changes?

It is clear that if we hope to gain a detailed understanding of the behavior of an enzyme in a complex biological system (such as an isolated organelle, intact cell, or entire organism) we must first attempt to understand its properties in as simple a system as possible. Thus, one cornerstone of metabolic biochemistry research has been the reductionist approach of enzyme purification and characterization. Enzyme purification eliminates metabolites and contaminating proteins that would otherwise confound kinetic and/or structural studies. By performing accurate and detailed analyses of the kinetic and regulatory properties of purified key enzymes in vitro and then combining these with knowledge of the enzyme’s subcellular localization and of the in vivo concentrations of the enzyme’s substrates, products, and metabolite effectors, one attempts to formulate a theory for the control of the enzyme in vivo. Such information can have broad practical applications and may be used to provide insights about various metabolic diseases, facilitate the identification of suitable targets for the disruption of a pathogen’s metabolism with drugs, or generate key information for biotechnologists wishing to manipulate specific metabolic pathways via metabolic engineering. From in vitro studies of a purified enzyme we can also learn about its structure, specificity for substrates, and reaction mechanism. With sufficient quantities of purified enzyme, structural biochemists can employ powerful methods such as chemical modification and covalent labeling, peptide isolation and sequencing, X-ray crystallography, mass spectroscopy, NMR spectroscopy, and so on, to determine the relationship between an enzyme’s structure and its function. This leads to the identification of key amino acid residues that are critical in substrate/product or allosteric effector binding, catalysis and covalent modification (i.e., phosphorylation) sites. Integration of biochemical (kinetic and structural data) and genetic information for a given enzyme ultimately provides a logical basis for site-directed mutagenesis by suggesting which amino acid(s) should be modified to produce a mutant enzyme with altered kinetic/regulatory properties.

Cautionary Note about Relating In Vitro Kinetic Studies of a Purified Enzyme to Its In Vivo Function and Control

The traditional approach to metabolic control outlined above is largely based upon the extrapolation of the in vitro kinetic/regulatory characteristics of purified enzymes to conditions within the intact cell. However, it is well-known that large multimeric regulatory enzymes such as PFK are susceptible to artefactual posttranslational modifications such as partial proteolysis by endogenous proteases (during purification), as well as dilution-dependent alterations in their oligomeric structure. Even very minor proteolytic clipping may obscure the allosteric properties of a purified regulatory enzyme, without markedly influencing its $V_{\text{max}}$. The diagnosis and prevention of unwanted proteolysis, through the addition of appropriate protease inhibitors to purification buffers, should be a major concern of all enzymologists.

The influence of protein concentration must also be considered because enzymes are present in vivo at far greater concentrations than they are during in vitro kinetic assays. Concentration dependence is believed to be particularly significant for enzymes important in metabolic control because their structure, and hence kinetic/regulatory properties, may be affected by protein–protein interactions. The interactions between enzyme subunits that normally exist at the high protein concentration prevailing in vivo can be specifically promoted in vitro by the addition of molecular crowding agents such as 10% (v/v) glycerol or 5% (w/v) polyethylene glycol to the reaction mixture. The mechanism involves exclusion of the protein from the aqueous solvent, thus increasing local enzyme concentration and favoring protein–protein interactions. The in vitro activity of many regulatory oligomers, including rat liver PFK, can be enhanced by the presence of such molecular crowding agents and can aid the examination of the catalytic and regulatory properties of enzymes in an in vitro environment that may be a closer approximation of the conditions prevailing in vivo.

Compartmentation of Metabolism

In the intact cell the individual enzymes of a metabolic pathway function to convert a starting material to end product(s) without necessitating the accumulation of elevated concentrations of the corresponding metabolic intermediates. In addition, there are many connections between the major metabolic pathways, with selected substrates, co-factors, regulatory molecules, and occasionally enzymes being common to more than one pathway. These complex
interactions can only be fully understood when, in addition to studying the isolated enzymes, some knowledge has been acquired concerning the intracellular location and concentrations of the enzyme and metabolites involved, and of any permeability barriers that separate the individual components. Thus, the functions of many enzymes cannot be fully understood without knowledge of their subcellular location.

The appearance of eukaryotic cells during evolution was associated with the process of compartmentation of metabolism through the formation of specialized organelles such as lysosomes, mitochondria, and plastids, separated from the cytosol and from each other by selectively permeable membranes. Compartmentation concentrates enzymes of a metabolic pathway and their associated metabolites, and prevents the simultaneous occurrence of potentially incompatible metabolic processes. The integration of cellular metabolism necessitates controlled interactions between pathways sequestered in the various subcellular compartments. This is facilitated by the existence of numerous membrane transporter proteins that selectively translocate specific metabolites between subcellular compartments. Thus, a major advance in the biochemical study of eukaryotic cells was the development of methods for separating intact organelles from the cytosol and from each other. This has not only allowed analysis of the overall biological functions of isolated organelles (i.e., respiration in mitochondria, photosynthesis in chloroplasts, etc.) but has also facilitated the determination of the distribution of enzymes and metabolites within cells, as well as the various metabolite translocators of the organelle membranes. Understanding these transport processes is of great importance for the overall understanding of metabolism and its control.

**Formulation of a Theory of Metabolic Control**

Once the controlling enzyme(s) of a given pathway have been identified and their kinetic/regulatory properties and subcellular localization investigated, it should be feasible to postulate a theory for the control of pathway flux. The theory should give rise to predictions that can be tested experimentally. This often necessitates collecting data about the activities and state of covalent modification (i.e., phosphorylation status) of the key enzymes, and the concentrations of their substrates and effectors under a variety of physiological conditions. It is important to demonstrate that these parameters reflect the in vivo situation as closely as possible. For example, is the in vivo ratio of a pacemaker enzyme’s [activators]:[inhibitors] proportional to pathway flux (i.e., following pathway stimulation, do the levels of the enzyme’s activators and inhibitors, respectively, increase and decrease, and vice versa)? Similarly, are the variations in the in vivo concentrations of effectors sufficient to account for the observed changes in enzyme activity or pathway flux? If not, then the initial theory must be revised and retested. This may require a more detailed investigation of the properties of the isolated enzyme.

One problem with the traditional pacemaker approach to metabolic control is that the studies are for the most part qualitative rather than quantitative. For example, the demonstration that a particular enzyme catalyzes a nonequilibrium reaction in vivo, shows a positive crossover during pathway activation, and exhibits pronounced control properties in vitro provides a unequivocal indication that it is relatively important in controlling pathway flux in vivo. However, designation of an enzyme as a pacemaker is not based upon any direct measurement of the precise contribution of each enzyme in a pathway to the overall control of pathway flux. Where more than one enzyme appears to be “regulatory,” there is no estimate as to their relative contributions, or how the degree of control exerted by each enzyme might vary under differing physiological circumstances. Furthermore, biological systems may display regulatory properties that are not possessed by their isolated components. In other words, the properties of biological systems tend to be greater than the sum of the properties of their isolated parts (i.e., the so-called Humpty-Dumpty principle). For instance, it would be impossible to understand how a mitochondrion functions in respiration by only studying purified mitochondrial enzymes and electron transport proteins in isolation from each other and from mitochondrial membranes. Thus, another important approach to the problem of metabolic control is to analyze the whole system.

**METABOLIC CONTROL ANALYSIS**

The metabolic control analysis (MCA) theory developed by Kacser in 1973 attempts to provide a quantifiable mechanism for probing intact biological systems and interprets the resulting data without preconceived notions as to which enzymes in a pathway are rate-determining steps or pacemakers (Fell, 1997). In fact, an important tenet of MCA theory is that metabolic control is shared among many, if not all, steps in a pathway. As discussed in more detail in a later chapter (and in references at the end of this chapter), Kacser has established the concept of the flux control coefficient \( C_i^f \) whose value specifies the change in metabolic flux \( (\Delta J) \) that results from a small change in the activity of any enzyme \( (\Delta E) \) in the metabolic system as follows: \( C_i^f = (\Delta J/\Delta E) \). For a linear pathway, the flux control coefficients of the component enzymes will lie between 0 and 1, with higher values indicating a greater contribution to metabolic control.
MECHANISMS OF METABOLIC CONTROL

TEXT BOX 1.1 THE PFK PARADOX

Phosphofructokinase (PFK) is generally considered to be an important pacemaker enzyme of the glycolytic pathway. It catalyzes the first unique step of glycolysis, a nonequilibrium reaction in vivo and shows a strong positive crossover concomitant with glycolytic stimulation. PFK is a multimeric enzyme that displays sigmoidal substrate (F6P) saturation kinetics as well as complex and potent allosteric regulation by numerous effectors, the levels which are controlled by the hormonal and/or nutritional status of the tissue. For example, the role of fructose-2,6-bisphosphate as a potent allosteric activator of animal and yeast PFK is well-established. However, the use of molecular genetic techniques for the selective overexpression of PFK in yeast, mammals, and plants has failed to yield significant increases in glycolytic flux or respiration that were expected. It appears that the elevated PFK concentration was somewhat compensated for in vivo by changes in the levels of PFK allosteric activators and inhibitors. PFK flux control coefficients were determined to be very small, leading to the surprising conclusion that PFK exerts very little or no control over glycolytic flux or respiration in vivo. Although proponents of MCA have challenged the traditional concept that PFK is a pacemaker enzyme of glycolysis, several MCA advocates have nevertheless agreed that there is little doubt that “control of PFK activity plays a part in glycolytic flux control” (Thomas and Fell, 1998) and “PFK makes an important contribution to the control of glycolysis in most cells” (Cornish-Bowden, 1999). A possible explanation for this “PFK paradox,” is that MCA also indicated that significant flux control of glycolysis and respiration lies in the metabolism of key feedback inhibitors of PFK, namely ATP and citrate in yeast and mammalian cells and phosphoenolpyruvate in plant cells. This would suggest the somewhat contradictory conclusion that, although the flux control coefficient for PFK may be low, it does indeed play an important role in the control of carbohydrate catabolism in most cells.

However, for complex pathways involving branches or substrate cycles, the flux control coefficients can hold any value, less than 0 or greater than 1. It should be emphasized that measurement of a flux control coefficient for a single step in a metabolic pathway may be difficult to interpret. The most satisfactory, yet highly challenging, way to apply MCA to a pathway would be to estimate the flux control coefficients for each component enzyme. As values for a single step may mislead.

Experimental determination of the magnitude of appropriate flux control coefficients apparently yields an unambiguous evaluation of the existing quantitative allotment of control among the various steps in a pathway, under specified conditions. Moreover, since the values of flux control coefficients can redistribute between enzymes according to physiological circumstances, any particular flux control coefficient applies only to the physiological state in which it was determined. Because metabolic control theory predicts that all enzymes in a pathway exert some control on pathway flux, all enzymes (in a linear pathway) should theoretically have flux control coefficients greater than 0. However, no single enzyme would be expected to have a flux control coefficient as high as 1.0 (which would be the case if pathway flux were entirely controlled by a single pacemaker enzyme). In fact, the summation theorem states that the sum of flux control coefficients for all component enzymes of a metabolic system should equal to 1.0.

The magnitude of any one flux control coefficient is not an intrinsic property of the enzyme per se but is a system property that depends upon the concurrent activities of all the other enzymes in the pathway. Thus, the value of a flux control coefficient cannot be determined by considering the properties of a purified enzyme since the characteristics and amounts of other enzymes in the system will influence the result. Individual flux control coefficients must therefore be determined experimentally from the intact system by measuring how pathway flux changes following alterations of the activity of a specific enzyme in situ. Advances in molecular biology now allow for direct manipulations of in vivo enzyme activities and continue to yield new information on the control of metabolism. There are a number of excellent publications and several Internet sites devoted to the quantitative MCA approach to metabolic control (see references). The various formulations and concepts of the mathematical models of MCA have given rise to considerable debate over the meaning and usefulness of flux control coefficients (see Text Box 1.1). However, most metabolic biochemists would likely agree that flux control of a metabolic pathway is generally dominated by a minority of its component enzymes (i.e., the pacemakers), although under different physiological conditions the degree of control exerted by the individual enzymes may vary.

MECHANISMS OF METABOLIC CONTROL

The magnitude of metabolite flux through any metabolic pathway will depend upon the activities of the individual enzymes involved. It is possible to group mechanisms of metabolic control into two major classes on the basis of the relative lengths of time they take to bring about a
change in the velocity of a particular enzyme. These are “coarse” and “fine” metabolic control.

Coarse Metabolic Control

Coarse metabolic control is a long-term (hours to days in eukaryotes; perhaps minutes to hours in rapidly growing prokaryotes), energetically expensive, response that is achieved through changes in the total cellular population of enzyme molecules. The total amount of a given enzyme is dependent upon the relative rates of its biosynthesis versus degradation. Thus, any alteration in the rates of gene expression [i.e., transcription, translation, messenger ribonucleic acid (mRNA) processing or degradation] or proteolysis can be considered as coarse metabolic control. Coarse control can be applied to one or all the enzymes in a particular pathway and most frequently comes into play in response to hormonal stimulation and/or during tissue differentiation or long-term environmental (adaptive) changes. The dynamic range of coarse metabolic control can be large, particularly when a previously absent enzyme is induced and rises to high levels in response to a stimulus. Coarse metabolic control might appear to be an inefficient and wasteful use of energy since each peptide bond formed requires the hydrolysis of several ATP equivalents, whereas protease activity is not coupled to the production of ATP (but as discussed below can also be ATP-dependent). However, coarse control may be particularly important when a cell must acclimate to changes in its environment, or it becomes necessary to remove abnormal enzyme molecules that have become damaged or arisen by errors in gene expression. In general, the longer the life of an individual cell the more important is the process of intracellular enzyme turnover. For example, in E. coli growing under optimal conditions, mitosis may occur every 20 min. Acclimation of E. coli to its environment occurs largely by the induction or repression of enzyme synthesis. For example, if lactose is added to the growth medium, rapid induction of β-galactosidase (needed to catabolize lactose) occurs. If lactose is then removed from the medium, the enzyme is not synthesized, and existing β-galactosidase molecules will be rapidly diluted out within the cytoplasm during the rapid division of cells. In contrast to rapidly dividing microbes, the average lifetime of a cell in a multicellular eukaryote may be several hundred days or more, but many enzymes need to be completely replaced every few days.

Gene Expression

The regulation of transcription and translation is covered in Chapters 6 and 7 and will not be discussed in detail here. However, in the context of metabolic control it is important to note that an underlying assumption of many genomic studies is that the expression of a gene at the mRNA level is a quantitative indicator of function of the encoded enzyme. Thus, an n-fold increase in transcript levels (detected via Northern blots or gene chip screening) equates to n-fold more enzyme and hence n-fold more activity. However, it is becoming apparent that this assumption does not always reflect reality. For example, a study that determined fluxes through steps of a central metabolic pathway (glycolysis) in three parasitic protists found that these did not correlate proportionally with the concentration of the corresponding enzymes; that is, relative to various fine metabolic control mechanisms, gene expression alone exerts little control on glycolytic flux.

Protein Turnover Relative to gene expression, much less is known about the mechanisms governing protein degradation. Animal and plant enzymes that coexist in the same cellular compartment may exhibit vastly different turnover rates, ranging from several minutes to hundreds of hours. In general, larger, oligomeric proteins that display complex biological properties and significant hydrophobicity tend to show much shorter half-lives in vivo than do less complex monomeric (and/or less hydrophobic) proteins. It is clear that proteolysis of enzymes can be selectively targeted and may be initiated in response to specific stimuli.

How are Enzymes Selected for Intracellular Degradation? Many enzymes need to first become “tagged” before becoming susceptible to degradation by endogenous proteases. The types of covalent modification used for tagging enzymes for degradation include the formation of a peptide bond between the target enzyme and a protein called ubiquitin, or the modification of the protein by phosphorylation or by oxidation. Ubiquitin (M, 9000) is so-called because of its widespread occurrence in eukaryotic cells. Its role in protein turnover has been well-established in animals and plants. Certain proteins destined for degradation are covalently bonded to ubiquitin via the NH2 groups of lysine residues. A single protein may become tagged with many ubiquitin molecules. ATP is required for the ubiquitin conjugation, together with several enzymes. Another method of tagging an enzyme for protease degradation is by phosphorylation, which again is dependent upon the hydrolysis of ATP, in this case by the modifying protein kinase (see below). The ATP requirement of the ubiquitin and phosphorylation tagging systems reflects the bioenergetic cost for endowing the cell with proteolytic specificity. Although tagging methods appear to make many enzymes susceptible to proteolytic attack in vivo, it is not yet clear precisely which features of the target enzymes are recognized by the tagging machinery.
Fine Metabolic Control

Fine metabolic controls are generally fast (i.e., seconds to minutes), energetically inexpensive, regulatory devices that modulate the activity of the preexisting enzyme molecule. Operating primarily on the regulatory or pacemaker enzyme(s) of a pathway, fine controls allow the cell to prevent metabolic chaos. Fine controls can be thought of as metabolic transducers that "sense" the momentary metabolic needs of the cell and modulate flux through the various pathways accordingly. It is important to note that the fine metabolic controls discussed in detail below are not mutually exclusive but often interact with, or may actually be dependent upon, one another.

Fine Control 1. Alteration in Substrate Concentration

The rate of an enzyme-catalyzed reaction is dependent upon [S] when [S] is subsaturating in vivo. Oftentimes, the in vivo [S] is less than or nearly equal to the $K_m$ or $S_{0.5}$ value of the enzyme for that substrate. The main exception to this are enzymes such as nucleases, proteases, lipases, phosphorylases, or amylases that catalyze initial steps in macromolecule catabolism, cases where substrate reserves (i.e., glycogen, triglycerides deposits) are huge.

Can pathway flux be controlled by alterations in [S] for any of the enzymes that comprise the pathway? Following stimulation of a metabolic pathway, the concentration of its constituent metabolites may increase severalfold (frequently 2- to 5-fold). However, pathway flux may increase by as much as 100-fold under the same stimulation. For enzymes that obey hyperbolic substrate saturation kinetics (i.e., Michaelis-Menten kinetics where Hill coefficient or $n_H = 1$) (Fig. 1.1a), Table 1.2 shows that the increase in [S] must be about 80-fold in order to obtain a 9-fold change in the activity of the enzyme. Such an increase in [S] is rarely if ever seen and so variation in [S] cannot be the sole determinant of the in vivo activity of enzymes that follow hyperbolic saturation kinetics.

Not all enzymes show simple Michaelis-Menten kinetics, however. Multimeric enzymes (i.e., consisting of two or more polypeptides in their native state) often contain more than one substrate binding site. Binding of substrate to one subunit can affect the conformation of other subunits and positively enhance the binding of substrate to them. The result of such cooperative binding of substrate is a sigmoidal relationship between enzyme activity and [S] (Fig. 1.1b). Enzymes of this nature have been termed allosteric because they can assume "other shapes" or conformations by the reversible, noncovalent binding of a specific metabolite. Sigmoidal substrate saturation kinetics has been referred to as homotropic allosterism since the allosteric modulator and the substrate are identical. Table 1.2 shows that the same 9-fold increase in activity that required an 80-fold increase in substrate concentration for a hyperbolic enzyme can be achieved with only a 3-to 4-fold increase in [S] for a sigmoidal enzyme (Table 1.2). The actual increase in [S] that is required is dependent upon the degree of cooperativity with which the enzyme binds its substrate. With increased cooperativity (i.e., higher values for $n_H$ reflected by increasing sigmoidal $V$ vs. [S] plots) smaller fold increases in [S] are required to give the same relative increase in enzyme activity.

In summary, changes in substrate concentrations that normally occur in vivo can alter the rate of pathway flux but do so most effectively for enzymes that show sigmoidal saturation kinetics (i.e., homotropic allosterism). Enzymes of this nature have been found in all phyla. Invariably such enzymes have been identified as pacemakers. Note, however, that not all multimeric or pacemaker enzymes necessarily display sigmoidal substrate saturation kinetics. Although sigmoidal kinetics allow a much more sensitive control of reaction rate by [S], it should be stressed that an alteration in [S] as a mechanism of fine control is often unimportant, relative to the other fine controls discussed below. The real metabolic "advantage" of sigmoidal substrate saturation kinetics is that this may allow metabolite activators (and/or covalent modification) to facilitate a marked enhancement in enzyme activity (at a relatively constant [S]) by promoting an allosteric transition that brings about a reversible shift between hyperbolic and sigmoidal saturation kinetics (see below).

Fine Control 2. Variation in pH

Most enzymes have a characteristic pH at which their activity is maximal, that is, the pH optimum. Above or below this pH the activity normally declines, although to varying degrees depending upon the particular enzyme. Thus, enzymes can show pH versus activity profiles ranging in shape from very broad
Figure 1.1 Relationship between substrate concentration and reaction rate for enzymes that follow (a) hyperbolic or (b) sigmoidal substrate saturation kinetics. $[S]_{90}, [S]_{50}$, and $[S]_{10}$ denote the respective substrate concentration that yields 90, 50, and 10% of $V_{max}$, respectively. To increase the activity of an enzyme that follows hyperbolic substrate saturation kinetics (a) from 10 to 90% of $V_{max}$, the increase in its $[S]$ must be about 80-fold. A much lower increase in $[S]$ is needed to achieve the same relative increase in the activity of an enzyme that follows sigmoidal substrate saturation kinetics (see text and Table 1.2 for details).

to very narrow. As the pH optimum of an enzyme is not always the same as the pH of its intracellular surroundings, this suggests that the pH dependence of enzyme activity may be one factor that determines its overall activity in the cell. As all cells contain thousands of enzymes, many of which show very different responses to pH, the intracellular pH ($pH_i$) may represent an important element of fine metabolic control.

The light-dependent activation of several of the enzymes of the reductive pentose-phosphate pathway (Calvin–Benson cycle) provides a well-documented example of how changes in $pH_i$ can contribute to metabolic control.