CHAPTER TWO

Mathematical Models of Folate-Mediated One-Carbon Metabolism

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Contents

Abstract

Folate-mediated one-carbon metabolism is an unusually complex metabolic network, consisting of several interlocking cycles, and compartmentation between cytosol and mitochondria. The cycles have diverse functions, the primary being thymidylate synthesis (the rate limiting step in DNA synthesis), the initial steps in purine synthesis, glutathione synthesis, and a host of methyl transfer reactions that include DNA and histone methylation. Regulation within the network is accomplished by numerous allosteric interactions in which

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metabolites in one part of the network affect the activity of enzymes elsewhere in the network. Although a large body of experimental work has elucidated the details of the mechanisms in every part of the network, the multitude of complex and non-linear interactions within the network makes it difficult to deduce how the network as a whole operates. Understanding the operation of this network is further complicated by the fact that human populations maintain functional polymorphisms for several enzymes in the network, and that the network is subject to continual short and long-term fluctuations in its inputs as well as in demands on its various outputs. Understanding how such a complex system operates is possible only by means of mathematical models that take account of all the reactions and interactions. Simulations with such models can be used as an adjunct to laboratory experimentation to test ideas and alternative hypotheses and interpretations quickly and inexpensively. A number of mathematical models have been developed over the years, largely motivated by the need to understand the complex mechanisms by which anticancer drugs like methotrexate inhibit nucleotide synthesis and thus limit the ability of cells to divide. More recently, mathematical models have been used to investigate the regulatory and homeostatic mechanisms that allow the system to accommodate large fluctuations in one part of the network without affecting critical functions elsewhere in the network.

I. Introduction

The origin of our mathematical modeling work stems from an interest in understanding how genes and the environment interact in the biochemistry of cells. This led us to study folate and methionine metabolism because this part of cell metabolism is linked to a diversity of human diseases that have both genetic and environmental contributing factors. Folate and other B vitamins play critical roles in the biochemical reactions of one-carbon metabolism that are related to amino acid metabolism, nucleotide synthesis, and numerous methyl-transferase reactions, including DNA and protein methylation.

Defects in folate-mediated one-carbon metabolism (FOCM; Table 2.1 lists the acronyms and abbreviation used in this chapter), either due to mutations in the genes that code for enzymes in the pathway or to deficiencies in vitamin cofactors, are associated with megaloblastic anemia, spina bifida and other neural tube defects, cardiovascular disease, increased sensitivity to oxidative stress, and a variety of neuropsychiatric disorders. FOCM is also involved in the etiology of colorectal and other types of cancer, and chemotherapeutic agents, such as methotrexate and 5-fluorouracil, target FOCM and play a central role in cancer treatment. FOCM is highly complex. It consists of a set of interlocked biochemical cycles (Fig. 2.1) whose enzymes are subject to complex allosteric regulations. The function of this complex network is further complicated by the fact that there are genetic polymorphisms for many of the enzymes in the network, and the functions

of the network are sensitive to the input of various amino acids (glycine, serine, methionine, and cysteine), B vitamins (folic acid, B_6 and B_{12}), and is affected by environmental factors such as alcohol intake in intricate ways that alter the normal operation of the network and the risk of disease.

Considerable research over the past 40 years has identified most if not all of the important details of FOCM. However, a limiting factor of these critical studies is that they have primarily focused on single reactions and on small portions of the pathway, and thus provide no means for understanding the overall functioning of the system. The multiple cycles and pathways of FOCM together are part of a complex nonlinear system, which is difficult to capture using purely experimental methods. Mathematical modeling is an approach that has been particularly useful in the study of complex biological systems (Edelstein-Keshet, 1988; Murray, 1989). Below we will review how mathematical modeling has been able to confirm key hypotheses about the operation of various portions of FOCM, and how modeling has provided novel insights into the properties and consequences of various regulatory mechanisms that stabilize portions of the network against environmental perturbations.

Table 2.1 Abbreviations and acronyms used in the text and figures

Acronym	Name
10f-THF	10-Formyltetrahydrofolate
10f0DHF	10-Formyldihydrofolate
5fTHF	5-Formyltetrahydrofolate (leucovorin)
5mTHF	5-Methyltetrahydrofolate
AICAR	P-ribosyl-5-amino-4-imidazole carboxamide
AICART	Aminoimidazolecarboxamide ribonucleotide transferase
BET, Bet	Betaine
BHMT	Betaine-homocysteine methyltransferase
CBS	Cystathionine β-synthase
CH:NHTHF	5-Formiminotetrahydrofolate
CH=THF	5–10-Methenyltetrahydrofolate
CH2-THF	5–10-Methylenetetrahydrofolate
CTGL	γ-Cystathionase
Cys	Cysteine
Cyst	Cystathionine
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHFS	Dihydrofolate synthase
DHPR	Dihydropteridine reductase
DMG	Dimethylglycine
DMGD	Dimethylglycine dehydrogenase
DNMT	DNA-methyltransferase

(*continued*)

Table 2.1 (*continued*)

Acronym	Name
dTMP	Deoxythymidine monophophate
dUMP	Deoxyuridine monophophate
FOCM	Folate-mediated one-carbon metabolism
FR-RFC	Folate receptor – reduced folate carrier
FTD	10-Formyltetrahydrofolate dehydrogenase
FTD	10-Formyltetrahydrofolate dehydrogenase
FTS	10-Formyltetrahydrofolate synthase
GAR	Glycinamide ribonucleotide
GCS	γ-Glutamylcysteine synthetase
GDC	Glycine decarboxylase (glycine cleavage system)
Glut	Glutamate
Glut-Cys	Glutamyl-cysteine
Gly	Glycine
GNMT	Glycine *N*-methyltransferase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione disulfide
$H_2C{=}O$	Formaldehyde
H_2O_2	Hydrogen peroxide
HCOOH	Formate
Hcy	Homocysteine
MAT-I	Methionine adenosyl transferase I
MAT-II	Methionine adenosyl transferase II
MAT-III	Methionine adenosyl transferase III
Met	Methionine
MS	Methionine synthase
MTCH	5,10-Methenyltetrahydrofolate cyclohydrolase
MTD	5,10-Methylenetetrahydrofolate dehydrogenase
MTHFR	5,10-Methylenetetrahydrofolate reductase
MTS	5,10-Methenyltetrahydrofolate synthetase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	non-enzymatic conversion
PGT	Phosphoribosyl glycinamidetransformylase
SAH	*S*-adenosylhomocysteine
SAHH	*S*-adenosylhomocysteine hydrolase
SAM	*S*-adenosylmethionine
Sarc	Sarcosine
SDH	Sarcosine dehydrogenase
Ser	Serine
SHMT	Serinehydroxymethyltransferase
TS	Thymidylate synthase
THF	Tetrahydrofolate

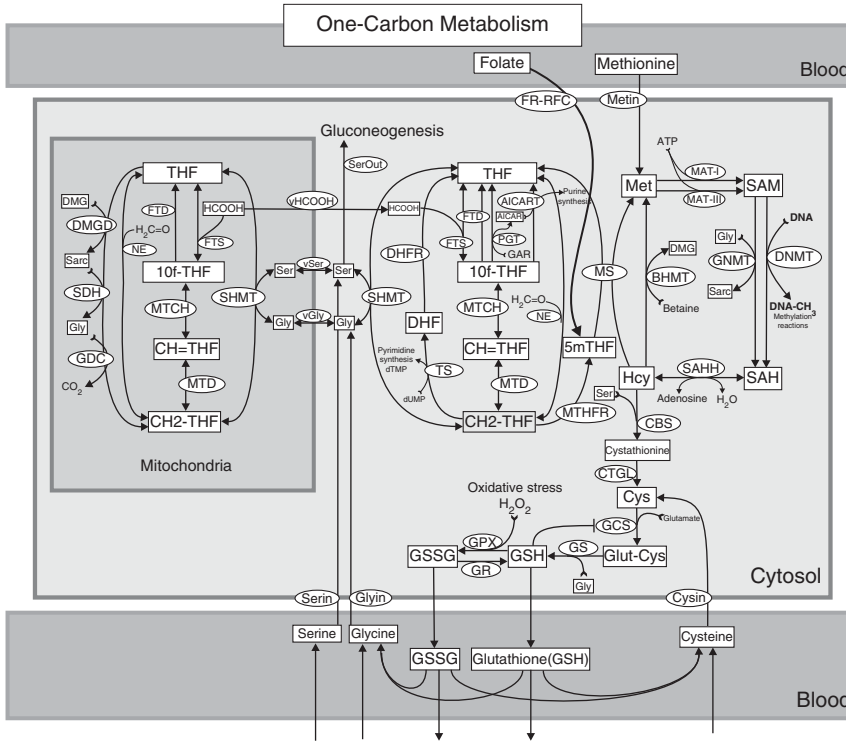

Figure 2.1 Diagram of mammalian hepatic folate-mediated one-carbon metabolism. This pathway includes the mitochondrial compartmentation, reduced glutathione synthesis, and transport of some metabolites from the blood. Metabolites that are variables in the model are enclosed in boxes, and enzymes are in ellipses. Full names of the acronyms of enzymes and metabolites are given in Table 2.1.

II. Structure and Function of the Cycles

FOCM consists of three functional modules: the folate cycle, the methionine cycle, and the glutathione synthesis pathway. In this chapter, we do not consider the role of the polyamine synthesis pathway which has recently been modeled by Rodriguez-Caso *et al.* (2006).

The function of FOCM is to pick up carbons from amino acids, primarily serine, but also glycine and methionine, and deliver them (as methyl groups) for the synthesis of purines and pyrimidines and for a variety of methylation reactions (e.g., DNA, tRNA, and histones) (Clarke and Banfield, 2001; Cook, 2001; Shane, 1995; Wagner, 1995). Serine enters the reactions as a substrate for SHMT which transfers one carbon to THF, yielding glycine and CH_2-THF. The glycine can enter the mitochondria where it is processed by the glycine cleavage system, transferring one of its carbons to CH_2-THF in the

mitochondrial folate cycle. The mitochondrial cycle then releases its carbons to the cytosol as formate (HCOOH in Fig. 2.1). Glycine is also used in the synthesis of sarcosine by GNMT. Sarcosine, in turn, also enters the mitochondria and eventually yields all but one of its carbons to the mitochondrial folate cycle. Serine is also used by the CBS reaction which complexes it with homocysteine to yield cystathionine, which, in turn, is used for the synthesis of cysteine and glutathione. The one-carbon units held by CH_2-THF have three fates: they can be passed to 5mTHF by MTHFR and subsequently to the methionine cycle where they are used in a great diversity of methylation reactions; they can also be passed to 10f-THF and subsequently used for purine synthesis; finally they can be passed to TS and used to synthesize dTMP from dUMP. Thus, FOCM plays a critical role in nucleotide synthesis, and the TS reaction is the rate-limiting step in DNA synthesis (Fukushima *et al.*, 2003). The cytosolic and mitochondrial SHMT reactions are reversible. In the forward direction they use serine, and in the backward direction they use glycine and one-carbon units from the mitochondrial folate cycle to synthesize serine, which can serve as the basis for gluconeogenesis. Methionine enters the methionine cycle and is adenosylated by MAT-I and MAT-III (in the liver; MAT-II is the adenosyl transferase used in other tissues). S-adenosyl methionine (SAM) serves as the general methyl donor for the majority of methylation reactions in the cell. About half of the mass of methionine that enters the methionine cycle leaves via the transulfuration pathway to cystathionine and cysteine (Finkelstein, 1990; Finkelstein and Martin, 1986), and the other half is remethylated to methionine by MS and BHMT, using methyl groups from 5mTHF and betaine, respectively.

If the reactions illustrated in Fig. 2.1 were the only pertinent ones, this would be a case of complicated but standard biochemistry. However, many of the metabolites in this system are allosteric activators or inhibitors of enzymes at some distance in the network. For example, SAM inhibits BHMT and MTHFR and activates CBS; DHF inhibits MTD, MTCH, and MTHFR; 5mTHF inhibits GNMT and SHMT (Finkelstein, 2003; Finkelstein and Martin, 1984; Finkelstein *et al.*, 1972; Jencks and Matthews, 1987; Kluijtmans *et al.*, 1996; Ou *et al.*, 2007; Yamada *et al.*, 2001; Yeo and Wagner, 1992). Many reactions also depend strongly on the cellular status for folate and vitamins B_6 and B_{12}. In addition, the velocities of many reactions depend on the concentrations of the substrates that are controlled by dietary inputs of glycine, serine, glutamate, cysteine and methionine. These inputs naturally undergo enormous fluctuations so the system is often far away from steady state (Nijhout *et al.*, 2007b).

It is a significant challenge to understand the biological reasons for the complicated interlocking cycles, the compartmentalization to the mitochondria, and the multiple reactions by which one substrate can be transformed into another. Since many parts of the network of FOCM share enzymes and metabolites, there must be mechanisms that ensure that large variation in a

particular region of the network does not compromise the function in other regions. Furthermore, the many critical reactions in the network must be buffered against large and irregular hourly and daily fluctuations in inputs of amino acids. The overall network is too complex to understand these regulatory functions by inspection of the reaction diagram alone, or to deduce the integration of its various functions with any degree of certainty. The system is well-enough understood that it is possible to develop a mathematical description of the kinetics of the various individual reactions, and couple these together into a single mathematical simulation model that can be used to explore questions about structure and function.

Over the past 5 years, we have developed mathematical models for the pathways shown in Fig. 2.1, which represents mammalian hepatic one-carbon metabolism. Hepatic FOCM is what we might call the ''complete'' pathway, in that all known enzymes and reactions operate in the liver. This is not true for most other tissues. While most FOCM enzymes are also expressed in the kidney, most other tissues in the body express only a subset of the enzymes and thus operate on what we might called ''reduced'' FOCM. FOCM is an ancient pathway and we have recently developed a model for the structure and kinetics of folate metabolism in bacteria (Leduc *et al.*, 2007 and Fig. 2.6).

III. Why Mathematical Modeling?

We view a mathematical model as an experimental tool, much like electrophoresis or PCR or gene knockout. Like all experimental tools, models have their own particular strengths and limitations and these should be understood if the tool is used to address a particular problem. A model is a mathematical description of a specific system. One of the particular strengths of a model is that it is completely explicit about what is in the system and what is not. In addition, a model is explicit about all the assumptions that are made about the properties of the components of the system and about their interactions.

The mathematical models we are dealing with here are not theoretical models in the sense that they attempt to discover necessary and sufficient conditions for the behavior of a particular system, or attempt to estimate parameter values for the system. Rather, they are strict quantitative descriptions of properties that have been determined experimentally by investigators. Ideally, a model represents the state of our understanding of the properties and interactions among the component parts of a system, and allows one to examine the behavior of the ensemble, and the consequences for the system as a whole of various assumptions one makes about how the components behave and interact. The model is ''tested'' against as much

experimental data as possible. Ideally, the model reproduces the results of a broad diversity of experiments both qualitatively and quantitatively.

If it does, then it can be used as an experimental tool to ask questions and do "experiments" that would be difficult, or expensive, or unethical to do in a real living system. In particular, a model provides a means to rapidly and inexpensively test the effects of specific perturbations and of alternative experimental strategies before committing time and resources to potentially expensive, and possibly inconclusive laboratory experiments. In its most useful guise, simulations with a model should interact with laboratory experimentation in a mutually illuminating exploration of FOCM.

A modeling approach is useful when the system one wishes to study is large and complex, with nonlinear interactions. Nonlinear systems produce context-dependent and nonintuitive responses to perturbations, and a simple examination of the connectivity diagram is seldom able to reveal anything useful about the dynamics of the system, nor its response to perturbation. FOCM is such a large, complex, and nonlinear system, consisting of several interlocking cycles with multiple inputs and outputs (Fig. 2.1). In addition, many of the enzymes in this system are subject to complex allosteric regulation by metabolites that are many steps removed in the network. These long-range regulatory interactions provide important homeostatic functions (see Section VII), which can only be evaluated by simulation studies.

A. Previous modeling efforts

A number of investigators have developed mathematical modes for various parts of FOCM (Harvey and Dev, 1975; Jackson and Harrap, 1973; Morrison and Allegra, 1989; Seither *et al.*, 1989; Vorontzov *et al.*, 1980; Werkheiser *et al.*, 1973) and the methionine cycle (Martinov *et al.*, 2000; Prudova *et al.*, 2005). Perhaps the best known among these are the extensive studies of R. C. Jackson and his associates (Jackson, 1980, 1984, 1986, 1993, 1995; Jackson and Harrap, 1973, 1979).

Almost without exception the models of folate metabolism have been aimed at understanding the mechanism of action and the kinetics of anticancer drugs, particularly methotrexate and 5-fluorouracil. In most cases the models focused on the portions of the system that were most relevant for their investigations. Jackson (1980, 1986; Jackson and Harrap, 1979) developed what is probably the most extensive model for folate metabolism (Fig. 2.2A), consisting of more than 60 reactions that also included the kinetics of membrane transport of folate and methotrexate, and more detailed reactions for the synthesis of purines, pyrimidines, RNA, and DNA. This model made specific predictions about the rates of DNA synthesis and the amount of time required to replicate all the DNA in a cell, and was thus able to estimate the maximal rate of cell division under

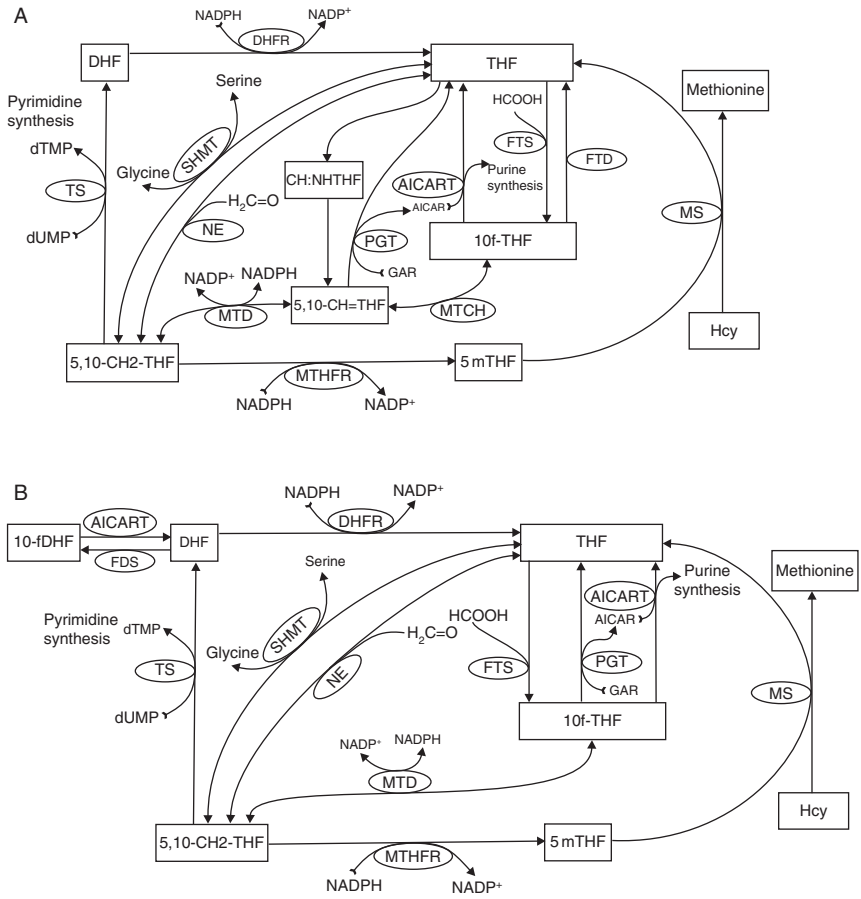

Figure 2.2 Diagrams of two early models of folate metabolism. A, the model of Jackson (1980). This model also includes synthesis of nucleotides, RNA and DNA, as well as the transport of folates and methotrexate into the cell, not shown in this diagram. B, the model of Morrison and Allegra (1989). Full names of the acronyms of enzymes and metabolites for this and other figures, and the text, are given in Table 2.1. Redrawn from Jackson (1980) and Morrison and Allegra (1989).

various methotrexate treatment regimes. The simulated results closely matched experimentally observed data on the inhibition of cell division by various methotrexate treatments. The Morrison and Allegra model (1989), shown in Fig. 2.2B, dealt specifically with the kinetics of folate metabolism in the MCF-7 breast cancer cell line, and included the effects of methotrexate polyglutamation and the consequent improved cellular retention of methotrexate. In spite of the fact that these models typically dealt only with subsections of one-carbon metabolism, and did not include any of the allosteric interactions that regulate and stabilize metabolite and fluxes,

they were generally able to simulate the correct pool sizes of several of the metabolites, and the time course of inhibition of nucleotide synthesis rates by treatment with antifolate cancer drugs.

B. Why modeling?

The traditional negative view of mathematical modeling is the following. If the biology and biochemistry are well understood, then there is no reason for models. On the other hand, if the biology or biochemistry is not well understood then there is not enough information to make an accurate model. Therefore, in either case, mathematical modeling is useless. And, of course, this negative view is reinforced by poor modeling or modelers who do not want deal with the full complexity of biological systems. In fact, many biological systems are ''partly understood'' in the sense that there is good information about many of the components of the systems but incomplete information about how the components work together to give rise to functional system properties. This is exactly the case with FOCM where a great deal of information is available on individual reactions but there is not much understanding of how the whole system works together. It is in this intermediate ''partly understood'' situation that a mathematical model can be a valuable, indeed necessary, investigative tool. To illustrate this point, it is helpful to face how difficult it really is to ''understand'' FOCM.

It is possible to understand a moderately sized traditional biochemical reaction diagram by walking the diagram. If substrate A goes up then, since A makes B, we expect B to go up and so forth. However, the existence of allosteric interactions by which substrates in one part of the network activate or inhibit enzymes in other parts makes this type of simple reasoning impossible or at best inconclusive. For example, SAM activates the enzyme CBS and inhibits the enzyme MTHFR. So, if we moderately increase the methionine input to the system, will the homocysteine concentration go up or down? Well, we would expect more mass in all the methionine cycle metabolites, so [Hcy] should go up. On the other hand, when methionine input goes up SAM rises appreciably and this will activate CBS, which will draw down [Hcy]. But since SAM is up, it will inhibit MTHFR, which will lower [5mTHF]. Since [5mTHF] is lower, the MS reaction will run more slowly and thus [Hcy] is not used as rapidly and thus should go up. So what will happen to [Hcy]? It is clear that no amount of verbal reasoning is going to answer this question, especially since the reactions and the allosteric interactions are nonlinear. One has to make calculations (and experiments) about the relative strengths of the competing influences on [Hcy]. Two other issues make the question even more daunting. First, the ''answer'' may depend on the overall context of the rest of FOCM (see below). Second, the allosteric activation of CBS and inhibition of MTHFR may

have evolved to stabilize [Hcy] concentration in the face of moderate changes in methionine input, in which case the answer to the question of whether [Hcy] goes up or down is "neither: it doesn't change much."

It is now well understood that gene expression is a stochastic process that leads to phenotypic protein differences even among "identical cells" (Elowitz *et al.*, 2002; Sigal *et al.*, 2006). Not only do the protein levels vary by as much as 15–30% from the mean from cell to cell, but also the levels vary over time even in individual cells. This variation has consequences for the "understanding" of FOCM. First, it does not make sense to ask for the exact value of a given parameter (a V_{max}, a K_m, or a K_i). Those values will vary substantially from cell to cell and from time to time in any given cell. Second, specific questions like "Does [Hcy] go up or down?" may have answers that depend on the context of all the other enzymes in the system. Third, some of the most important properties of FOCM (or indeed of all of cell metabolism) are regulations, not obvious from the standard biochemical reaction diagram, that allow the system to function despite these large variations. The allosteric interactions mentioned above are examples of such regulations. Thus, FOCM should not be thought of as a single fixed system but a whole family of systems with large variations in important parameters. It is difficult to see how one could understand "function" in the face of such variation without mathematical modeling.

In Nijhout *et al.* (2007b), we show how many of the concentrations and reaction velocities of hepatic FOCM react to the daily inputs of amino acids due to meals. Some concentrations and velocities fluctuate wildly while others are protected by regulatory mechanisms. More recent calculations with the full model depicted in Fig. 2.1 (see Fig. 2.10) show the same behavior. This is the reason, of course, that many experimental and clinical measurements are done in the "fasting state." Because of the difficulty of making many simultaneous measurements of concentrations and velocities as functions of time in living cells it is difficult to see how such dynamic fluctuating behavior could be investigated experimentally. Thus, mathematical modeling has a central role in elucidating the regulatory mechanisms that allow cells to adapt to such dramatic changes in inputs.

C. Difficult issues in modeling

Suppose that one wants to investigate a particular phenomenon in FOCM seen experimentally or clinically, for example, the behavior of homocysteine under methionine loading or the stability of the glutathione pool in the face of daily meals. Which variables and interactions should be included? If the model is too small, one may have excluded (or rather held constant) just those variables and interactions that are crucial to understanding the phenomenon. If the model is too large it may be too unwieldy to experiment with and the "noise" from all the approximations that one makes may

obscure the phenomenon that one wants to study. So, how large should a model be? This is always a difficult question (though usually not admitted by modelers), and every modeling attempt answers it explicitly by what is included and what is excluded. Since our goal is not only to reproduce experimental or clinical results but also to use the model to understand how and why they arise, our philosophy is to start with smaller models and expand to larger models when the smaller ones are well understood and the expansion to more variables is necessary. Thus, as we outline below in Section IV, we began with a model of methionine metabolism that had only four variables (Reed *et al.*, 2004). Then we made a model of the folate cycle (Nijhout *et al.*, 2004) so we could study the inhibition of DHFR by methotrexate and the allosteric binding of folates to folate enzymes. Then we made a larger model combining to two smaller ones so that we could study the effects of the inhibition of MTHFR by SAM and the inhibition of GNMT by 5mTHF (Nijhout *et al.*, 2006). There has been a long discussion in the literature or the role of the folate cycle in mitochondria (Appling, 1991; Christensen and MacKenzie, 2005) so to investigate these questions we added compartmentation and the mitochondrial reactions (Nijhout *et al.*, 2007a). At each stage we had to make difficult (imperfect) decisions about which variables and interactions to include in the models.

We note that this difficulty of knowing where to draw the boundaries is also a difficulty for the interpretation of laboratory experiments or clinical observations. In an experiment one changes the system by, say, knocking out a gene or introducing a chemical that binds to a particular enzyme. One then measures the changes in a few variables (the ''results'' of the experiment) and then makes conclusions about how the system functions. Implicitly, one is assuming that everything else besides what one measures is the same (or can be considered the same), that the knocked out gene did not affect other genes or that the inhibitor has no other effects but the intended one. The interpretation of the experiment typically involves implicitly drawing the boundaries of a ''model'' (in the experimenter's head) of which variables are allowed to be included in the interpretation. Thus, the interpretation of experimental results must always face and answer (albeit implicitly) the same difficult question of boundaries faced explicitly in modeling.

The next difficulty is deciding what level of detail to include for individual reactions in FOCM. An enormous amount of information is available about enzymes, their genes and conformations and the way that they bind to substrates. How much of this detail should be included? Our approach is to use simple Michaelis-Menten kinetics and simple kinetic forms for activations and inhibitions unless we have good reason to believe that a more detailed treatment is necessary for important biological functions of FOCM. For example, we could have modeled the synthesis of SAM from methionine in liver cells by a simple Michaelis-Menten formula. But we knew from the

experiments of Finkelstein *et al.* (1982) and Finkelstein and Martin (1984, 1986) that the methionine levels are fairly stable under methionine loading whereas SAM increases enormously, and that Corrales *et al.* (2002) had suggested that this is a result of the different kinetics of the two isoforms MAT-I and MAT-III. Because we believe that the stabilization of methionine and the many regulations by SAM are biologically important, we decided to include the rather complicated special kinetics of MAT-I and MAT-III in the model. Subsequently, we were able to show (unpublished) that the suggestion of Corrales *et al.* (2002) was completely correct.

Finally, of course, one must choose V_{max}, K_m, and K_i values. This is not such an easy matter since there are few measurements of V_{max} values and the reported measurements of V_{max}, K_m, and K_i values show large variation. Given the stochastic variation in gene expression discussed above and the dependence of protein conformation and function on the *in vivo* context, this variation is not surprising. We try to choose K_m, and K_i values within reasonable experimental ranges and adjust the V_{max} values so that the values of the metabolite concentrations are in the experimental ranges. It is always a question whether the results of *in silico* experiments would have been different if we had chosen different parameters. We have found that most of our qualitative results are quite insensitive to variations in parameter values. In some sense, it has to be that way because FOCM must have evolved to be able to continue to function in the face of the stochastic variation in gene expression discussed above. Nevertheless, all three difficulties that we have discussed necessarily temper the confidence that one has (that we have!) in model results.

D. Advantages of mathematical models

Although models have difficulties and limitations, they also have advantages and it is worthwhile to state them explicitly. First, to formulate a model one has to be explicit about one's assumptions. If A inhibits B one must say how much B is inhibited at different concentrations of A and how this may or may not depend on other variables in the system. Secondly, once one has a model, *in silico* experimentation is cheap, fast, and easy. One does not need animals, IRB protocols, or technicians. Third, and most important, when the model behaves in the same way as interesting experimental results, one can take the model apart and put it back together (by removing reactions or inhibitions, for example) until one understands the causal chain of events that gives rise to the observed behavior. Thus, experiments with the model can give real biological understanding of the phenomena under study. Finally, in the model, one can follow the time course of all concentrations and velocities and determine how the system reacts to outside influences or changes in internal parameters. This is impossible to do by *in vivo* experimentation.

Every experimental scientist is a modeler because every hypothesis is based on a conceptual model of how a system ought to behave. A mathematical model is simply a way of making a conceptual model explicit by describing and connecting all the underlying knowledge and assumptions. If a mathematical model does not reproduce the known behavior of a system then, obviously, the model is wrong. But if the model is based on all known data, then the ancillary conclusion is that the knowledge of the system must be inadequate. Thus, a model can reveal the inadequacy of current data or concepts. The model can then be used to test hypotheses about what kind of additional (or different) information can yield the correct behavior, and this can stimulate research to verify those predictions.

Another important use of a model is to test hypotheses about mechanisms that are difficult to study experimentally. We will give two examples from FOCM. The first comes from a series of studies by Finkelstein and Martin (1984, 1986) and Finkelstein (1990, 2001) who studied the allosteric effect of SAM on the CBS and BHMT. They suggested that the concentration of SAM rose with methionine input and that the allosteric stimulation of CBS and inhibition of BHMT by SAM would result in an increased transsulfuration of homocysteine, which removes the excess methionine from the system. Thus, the allosteric regulations by SAM constitute a homeostatic mechanism that stabilizes the mass in the methionine cycle. Our simulations with a model of the methionine cycle (Reed *et al.*, 2004) show that variation in methionine input is completely absorbed by variation in the concentration of SAM. The model also shows that the allosteric regulation of BHMT and CBS by SAM increases the transsulfuration rate in such a way that total mass in the methionine cycle, and the flux around the methionine cycle, remain stable in the face fluctuating methionine input, as first hypothesized by Finkelstein and Martin (1984).

The second example involves the role of the mitochondrial bifunctional enzyme. In the mitochondria, the MTD + MTCH reactions are catalyzed by a single bifunctional enzyme (Mejia and MacKenzie, 1986; Peri *et al.*, 1989). This enzyme is not normally expressed in adult cells; it is expressed only during embryonic development and in cancer cells (Di Pietro *et al.*, 2004; Smith *et al.*, 1990), so its expression appears to be restricted to cells undergoing high rates of cell division. On the basis of interpretation of a series of radiotracer and gene knockout experiments, Christensen and MacKenzie (2005) hypothesized that the bifunctional enzyme provides a "metabolic switch that controls the flow of one-carbon units to determine, for example, the degree to which mitochondria produce formate and/or convert glycine to serine." This hypothesis was confirmed by our mathematical model (Nijhout *et al.*, 2007a). Elimination of the mitochondrial bifunctional enzyme in the model did not show a runaway accumulation of CH_2-THF, as might be expected. Instead, the GDC reaction slowed down, the production and export of formate stopped entirely, and most

importantly, the mitochondrial SHMT reaction reversed direction and now ran toward serine synthesis. Thus, in the presence of the bifunctional enzyme, a situation typical of embryonic and cancer cells, the mitochondria export large quantities of formate that are directed to purine and TS in the cytosol. When the bifunctional enzyme is not expressed, as in adult cells that do not divide, the mitochondrial reactions become strong producers of serine, which is exported to the cytosol and where it is directed toward gluconeogenesis and other reactions. The bifunctional enzyme switch in effect transforms the mitochondria from formate factories into serine factories, and may thus be an adaptation to the very different metabolic and biosynthetic needs of rapidly growing embryonic cells and more quiescent adult cells, as suggested by Christensen and MacKenzie (2005).

E. Kinetics, parameter values, and model structure

The reported diversity of parameter values for the same enzyme can be due to various reasons: (1) the orthologous enzymes from different species can have different kinetic properties; (2) enzyme expression differs in different tissues, in particular some enzymes are up-or downregulated in cancers as well as in tissues of animals undergoing chronic nutrient or vitamin deprivation or excess; (3) different semipurified enzyme preparations may contain different, and unknown, concentrations of allosteric activators or inhibitors; (4) enzyme preparations made at different times of day can contain different concentrations of metabolites and allosteric effectors; (5) in bimolecular reactions the values of the K_ms depend on the concentration of both substrates (Segel, 1975), but it is common to maintain one of the substrates constant, resulting in the measurement of an apparent K_m that can differ depending on the preparation used.

Our approach to modeling the kinetics of one-carbon metabolism is to restrict our use of reported kinetics to those measured in mammals, preferably humans, and we differentiate between parameters measured in different tissues by building different models that specifically deal with hepatic one-carbon metabolism and epithelial one-carbon metabolism (Figs. 2.3 and 2.5). Although measures of kinetic parameter values can vary significantly, fortunately metabolite concentrations can be measured with great accuracy and consistency, and the actual flux through a particular reaction, or the relative dimensions of the fluxes through different portions of the pathway are sometimes known. We start our modeling by choosing a value of each K_m and K_i roughly in the middle of the reported range, and vary the V_{max} to obtain the reported metabolite concentrations and fluxes. We have found experimental V_{max} values to be largely not useful for modeling purpose since they are typically reported in units of rate/mg protein, without stating how protein was determined. We use values of the k_{cat} in those few cases where *in vivo* enzyme concentrations are known. We have

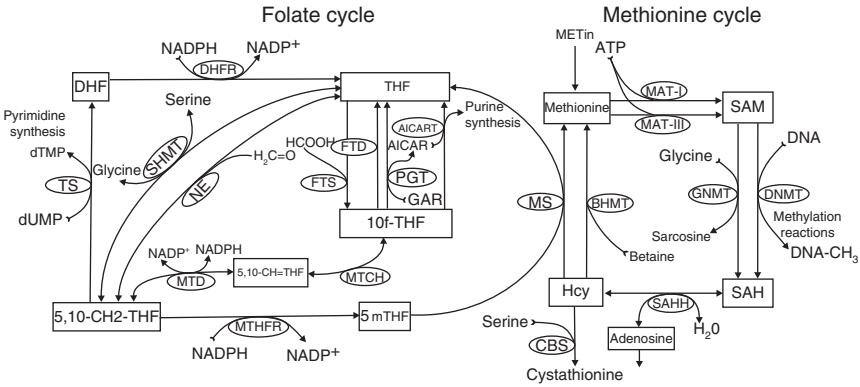

Figure 2.3 Diagram of our model of the coupled hepatic folate and methionine cycles. Not shown in this figure are all the allosteric interactions between metabolites and various enzymes in the two cycles. Full names of the acronyms of enzymes and metabolites for this and other figures, and the text, are given in Table 2.1. Redrawn from Reed *et al.* (2006).

found that the choices of V_{max} values are often constrained by the requirement that the model produce the right combination of known metabolite concentrations, relative flux rates, half-lives, and time-dependent responses to perturbations in the experimental literature.

When the kinetic mechanism of an enzyme is known we use the conventional equations for the relevant uni- or bimolecular reaction as described by Segel (1975). Allosteric activation or inhibition of enzymes often does not admit to one of the conventional equations. In such cases, we do a nonlinear regression on the published experimental data and use that as the empirical equation. In these, as in all, cases, we ensure that the model operates within the limits of the experimental data. Our models assume that certain substrates (dUMP, GAR) and energetic metabolites (ATP, NADP, and NADPH) are constant, so that their effect is absorbed by the V_{max} for the reaction.

At present the model does not contain terms for polyglutamation and deglutamation. The model also does not contain a nuclear compartment, although it is known that nuclear compartmentation is important (Appling, 1991; Woeller *et al.*, 2007). We set the total folate level in the cell by defining the overall size of the folate pool. If we start the simulation with all folates in one form (e.g., THF or 5mTHF), the reaction kinetics rapidly redistribute the folates, and the system comes to equilibrium for the different folate species in ~5–6 h. The half-life for folate in the body is about 90 days, and the mean residence time for folate is 124–212 days (Gregory and Quinlivan, 2002; Gregory *et al.*, 1998), so for short-term studies like the ones we do, the assumption of a constant intracellular folate pool seems reasonable.

The model then, consists of a set of kinetic formulas, one for each enzyme, that describe the velocity of the reaction as a function of the concentrations of substrates, products and allosteric regulators, plus a set of differential equations, one for each variable metabolite, that contain the kinetic formulas for its synthesis and degradation. In addition, we have transport functions for amino acids into and out of the cell, and or amino acids and formate into and out of the mitochondria. The overall system is solved by numerical integration using a stiff ode solver (because different quantities tend to vary at very different rates), implemented in MatLab (The MathWorks). The program allows us to vary inputs of amino acids and vitamins over time and follow the time-dependent responses of all metabolites and reaction rates. In addition, the model allows us to simulate the effects of mutations and of vitamin deficiency (or excess). We have modeled mutations primarily by altering the V_{max} values of the relevant enzymes. This would correspond to mutations that affect the amount of active enzyme present (e.g., mutations that affect enzyme expression or activation). Likewise, we model the effects of variation in non-folate vitamin cofactors, such as B_{12} and B_6, by altering the V_{max} of the corresponding enzyme(s), assuming in effect that the activity of the enzyme is a function of the amount of cofactor available.

IV. Model Development

Previous models of folate metabolism, outlined above, were developed in the 1970s and 1980s. Much new information and understanding have become available in the intervening 25 years, which have guided our approach. We began by first developing a model for the methionine cycle (Reed *et al.*, 2004). This model built on the prior work of Martinov *et al.* (2000) who had studied the properties of a model for a portion of the methionine cycle that did not include the MS, BHMT, and CBS reactions and used simplifying assumptions about inputs into the cycle. Our model closed the cycle and added the CBS reaction and several allosteric effects of SAM. This model was able to reproduce the observed dependence of the transsulfuration reaction on the concentration of SAM described by Finkelstein and Martin (1984), and the effects of variation in CBS and MS activity on homocysteine, methionine, and SAM (Finkelstein, 1990; Finkelstein *et al.*, 1974; Janosik *et al.*, 2001; Pogribna *et al.*, 2001; Rosenblatt, 2001). Perhaps the most interesting finding with this model was that SAM acts as a buffer for methionine input: that is, variation in methionine input has little effect on the methionine and homocysteine concentrations but is mostly absorbed by variation in the concentration of SAM. Furthermore the allosteric effect of SAM on CBS provides a

mechanism for stabilizing mass in the methionine cycle so that the flux out of the methionine cycle via CBS matches the rate of methionine input into the cycle without much change in the homocysteine concentration. If it were not for the allosteric effect of SAM, the homocysteine concentration would have to rise to drive the CBS reaction.

Our next step was to develop a model for the folate cycle that contained what at the time we understood to be the important reactions of that metabolic network (Nijhout *et al.*, 2004). This model incorporated the finding that folates bind to and inhibit many of the enzymes in the folate cycle. This binding was believed to provide a reservoir of folates. The model allowed us to resolve the puzzle as to why enzymes of the folate cycle should be inhibited by allosteric binding of folates. The model shows that this nonenzymatic binding greatly reduces the sensitivity of the system to folate deficiency, because as the total pool of folate diminishes, more enzyme is released from inhibition, and the reaction velocities are maintained because of the increased enzyme activity (Nijhout *et al.*, 2004). We next modeled the allosteric interactions between the folate and methionine cycles (Fig. 2.11) in order to test the hypothesis of Wagner *et al.* (1985) that these interactions serve to stabilize the DNA methylation reaction rates (Nijhout *et al.*, 2006). Some results of these experiments are outlined in Section VII below. We subsequently merged our models for the folate and methionine cycles (Fig. 2.3) to produce an integrated model of one-carbon metabolism (Reed *et al.*, 2006). This model also incorporated allosteric interactions between the folate and methionine cycles (inhibition of MTHFR by SAM and SAH, and inhibition of GNMT by 5mTHF) and added the ability to vary the rate of input of betaine. We used this model to simulate the interaction between folate deficiency and the MTHFR C677T polymorphism and the interaction between folate and vitamin B_{12} deficiencies. Experimentation with this model showed that the inverse relationship between folate status and homocysteine level is strongest at low folate levels and disappears at high folate levels. Furthermore, the model shows that as folate levels in the cell rise, the reactions of the folate cycle slow down. This is due to the allosteric inhibition of enzymes in the folate cycle by folate metabolites. This is a consequence of the homeostatic mechanism described by Nijhout *et al.* (2004). This mechanism stabilizes the folate cycle at low and intermediate folate levels, but also predicts that as folate levels rise, the reaction rates in the folate cycle will slow down. Thus, a prediction of the model is that a high intracellular folate level can have the same effect as a folate deficiency. This prediction of the model is now supported by a variety of clinical and experimental data that show that high doses of folate can have detrimental effects (Akoglu *et al.*, 2001; Czeizel, 2004; Morris *et al.*, 2005; Sunder-Plassman *et al.*, 2000; Troen *et al.*, 2006).

We then expanded the model of Reed *et al.* (2006) to include compartmentation of the folate cycle between cytosol and mitochondria

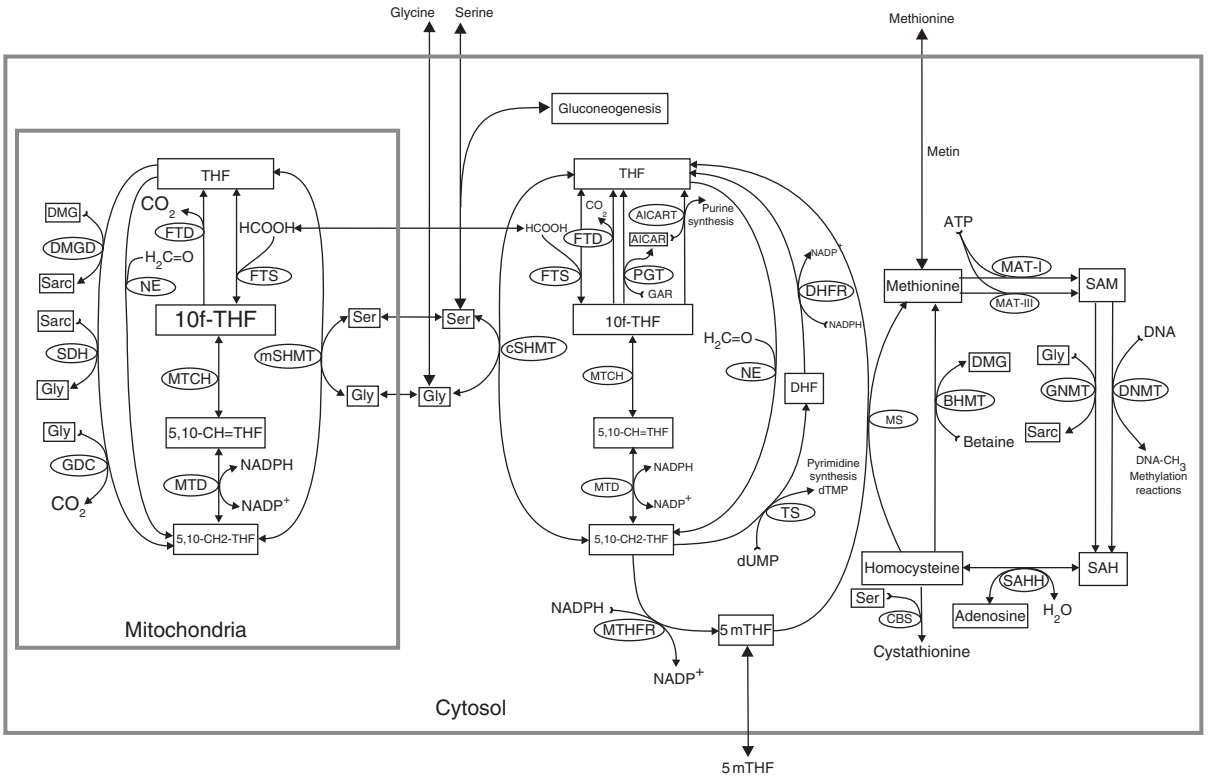

Figure 2.4 Diagram of our model of hepatic FOCM including the mitochondrial compartmentation. Boxed substrates are variables in the model. Substrates without boxes are constants. Full names of the acronyms of enzymes and metabolites for this and other figures, and the text, are given in Table 2.1. Redrawn from Nijhout *et al.* (2007a).

(Nijhout *et al.*, 2007a). This model included terms for the glycine cleavage system and the metabolism of sarcosine and dimethylglycine in the mitochondria, mechanisms for transport of serine and glycine into the cell and between the cytosol and mitochondria, and terms for the transport of formate between cytosol and mitochondria (Fig. 2.4). As discussed above, we discovered that in rapidly dividing cells mitochondria act primarily to supply formate to the cytosol for purine and pyrimidine synthesis, whereas in adult cells the mitochondria export no formate but are excess producers of serine, targeted for gluconeogenesis. We also found that the rate of export of formate from the mitochondria to the cytosol is remarkably insensitive to fluctuations in serine and glycine input. This is because both mitochondrial and cytosolic SHMT reactions are reversible and the rates at which they run are highly responsive to the relative concentrations of glycine and serine. The model was used to investigate the effect of varying the relative inputs of glycine and serine on the rate and direction of the mitochondrial and cytosolic SHMT reactions, and showed that both SHMT reactions can reverse and run in the serine synthesis direction when external glycine is increased replicating the results of Kastanos *et al.* (1997). This model was also used to successfully simulate the experiments of MacFarlane *et al.* (2005) and Herbig *et al.* (2002) on the effect of SHMT expression and glycine availability on SAM.

To investigate the characteristics of FOCM in nonhepatic tissues we developed a model for epithelial FOCM, which is representative of most tissues except liver and kidney. Extrahepatic tissues do not express all enzymes of FOCM, and some enzymes are active at much lower levels than in the liver (dashed arrows in Fig. 2.5). Epithelia thus run on a reduced version of the network. This model also includes a term for export of homocysteine, which is typically exported from extrahepatic tissues for remethylation in the liver. With this model we have explored the interaction of multiple genetic polymorphisms and the interaction of genetic and environmental variation on the level of homocysteine, the rates of methylation, and purine and pyrimidine synthesis (Ulrich *et al.*, 2008). We have also created a model (Fig. 2.1) that includes the synthesis of reduced glutathione and exchange of substrates with the blood (Reed *et al.*, 2008).

FOCM is an ancient pathway and occurs, with variations, in animals, plants, fungi, and bacteria. Recently, we have developed a model of bacterial FOCM for *Rhodobacter capsulatus* (Leduc *et al.*, 2007), motivated by the discovery of a novel flavin-dependent thymidylate synthase (ThyX) that produces THF rather than DHF upon methylation of dUMP (Fig. 2.6). This model was used to examine the relative roles of ThyA (TS), ThyX, and FolA (DHFR) in the mechanism of resistance to antifolates such as trimethoprim.

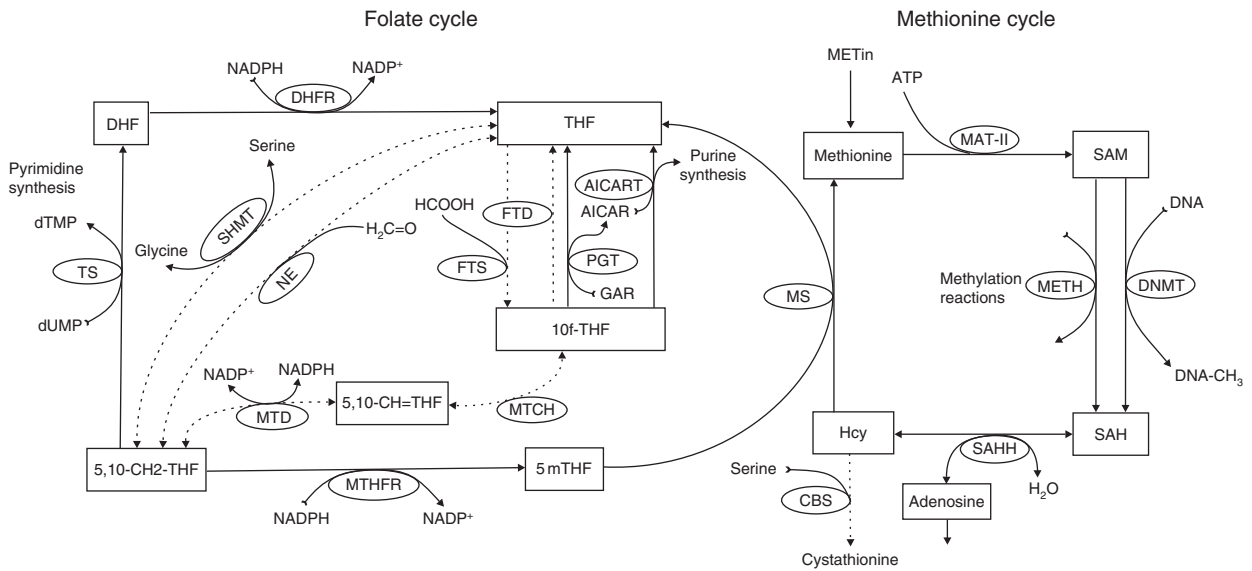

Figure 2.5 Diagram of our mode of epithelial FOCM. This model does not include mitochondrial compartmentation, but does include all allosteric regulations (not shown in this diagram). Dashed arrows indicate enzymes of low activity in epithelial tissues. Full names of the acronyms of enzymes and metabolites for this and other figures, and the text, are given in Table 2.1.

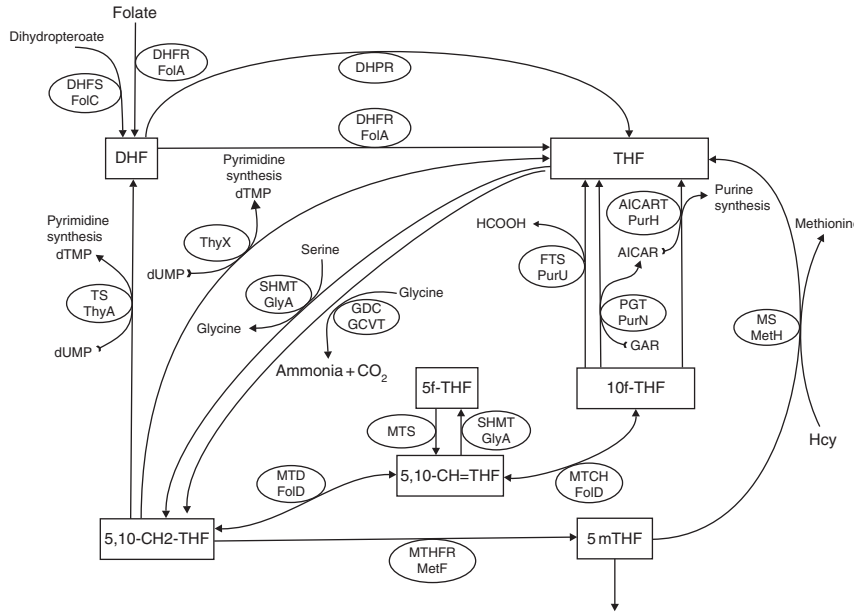

Figure 2.6 Diagram of our model of bacterial folate metabolism. This mechanism can synthesize thymidylate but bypass DHF. Redrawn from Leduc *et al.* (2007).

V. Blood Versus Intracellular Metabolite Concentrations

The models we have developed are for intracellular metabolism, and thus deal with intracellular concentration and pool sizes. However, almost all of our understanding of the relationships between folate status and disease is based on measurements of the concentrations of folate, homocysteine, SAM, SAH, and methionine in the blood, plasma, or red blood cells. Red blood cell measurements are believed to reflect the metabolite status at the time the red blood cells differentiated: in a mixed-age population of cells this presumably represents an average or long-term metabolic status. Blood and plasma concentrations may be in equilibrium with overall cellular cytosolic concentrations, though it is more likely that they result from the interaction between uptake from the digestive system, export by some tissues (like epithelial cells, kidneys, muscle, and nervous system), import by others (like the liver), and excretion by the kidneys. Whether these processes are ever a steady state is an open question. The half-life of folate in the body is about 90 days, and about 500 days are required for folate levels to come to a new steady state (Gregory *et al.*, 1998). Methionine loading experiments shows that the methionine and homocysteine levels in the blood require 12–24 h to return to steady state after a perturbation (Bianchi *et al.*, 2000;

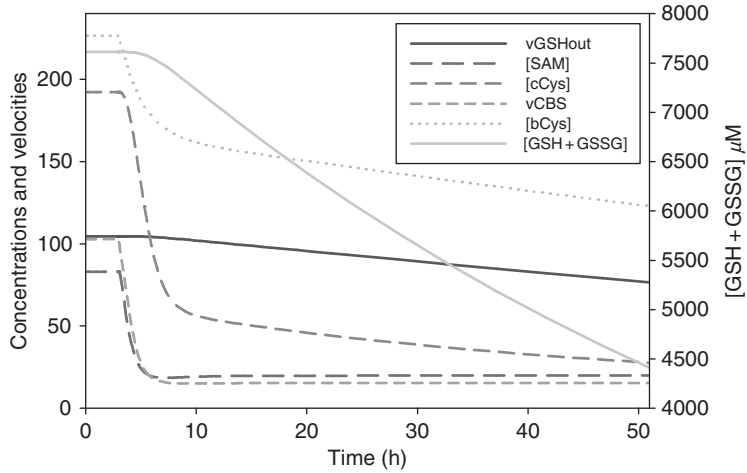

Figure 2.7 Response of selected metabolite concentration and reaction velocities to fasting. The system was allowed to come to steady state, and at 3 h the input of amino acids (glycine, serine, methionine, and cysteine) was reduced to 0.25 of their normal values. Different components of FOCM decline at different rates to a new steady state. Some approach steady state after 5–6 h, other take more than 48 h to approach the new steady state.

Silberberg and Dudman, 2001). In our models, the time required for different components of the system to relax to equilibrium is in the order of hours to days (Fig. 2.7). Given that variation in input into the system is in the order of hours, it is unlikely that the system is ever at steady state and may actually exist far from equilibrium most of the time (Nijhout *et al.*, 2007b).

Thus, blood measurements represent some average of what is going on in different cell types, and one would therefore expect a variable and context-dependent correlation between blood components (particularly for metabolites that are used in many processes) and the state of a given organ or cellular metabolic system. Our current intracellular models accurately simulate intracellular responses to experimental or clinical intervention, and it is obviously desirable for a model to also simulate how the levels of commonly measured blood metabolites will respond. We are beginning to approach this difficult question of whole body modeling of folate metabolism by allowing our cytosolic models for hepatic and epithelial FOCM to communicate with a blood compartment that is subject to dietary input and excretory output.

VI. Modeling Gene–Gene and Gene–Environment Interactions

One advantage of models is that they can be used to investigate the effects of simultaneous variation of many variables. In the case of FOCM, the models can be used to study the effect of simultaneous genetic

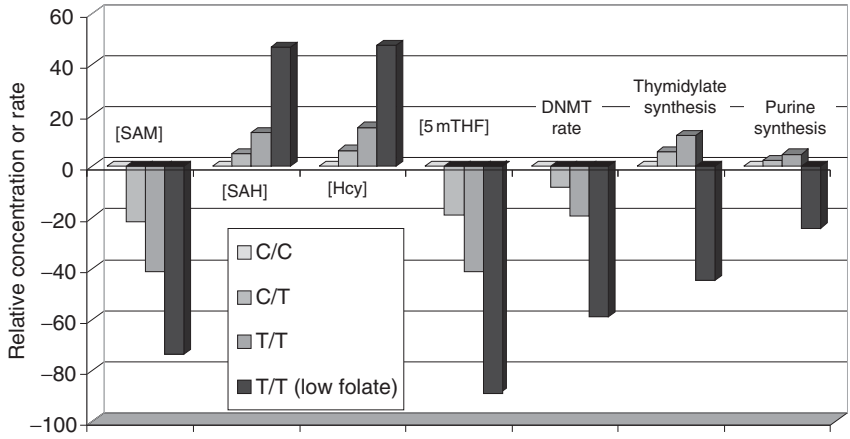

Figure 2.8 Simulated response of various biomarkers to the MTHFR 677CT polymorphism. Folate deficiency exacerbates the effect of the T/T genotype for most biomarkers, but reverses the effect of the T/T genotype on purine and pyrimidine synthesis.

polymorphisms, or the interaction between a polymorphism and an environmental variable such as an amino acid, vitamin B_{12}, or folate. For instance, the interaction of the MTHFR C677T polymorphism with low folate status is shown in Fig. 2.8. The T/T genotype is known to diminish the risk of colon cancer under high folate, but it enhances risk for cardiovascular disease (Curtin *et al.*, 2004; Frosst *et al.*, 1995). In the model, the T/T genotype lowers the concentration of SAM and the DNMT reaction rate but raises homocysteine levels and both thymidylate and purine synthesis rates. Folate deficiency enhances the effects of the T allele on most biomarkers, with the exception that it reverses the effect on thymidylate and purine synthesis. Although these simulated changes in biomarkers correspond to those observed in practice, it is not yet clear how these metabolic effects translate into differential risks for colon cancer and cardiovascular disease.

The interaction between variation in the V_{max} of MS and of MTHFR is illustrated in Fig. 2.9. The variation along the MS axis in Fig. 2.9 can be interpreted in several ways. It can represent variation in the expression level of MS which could be due to a regulatory mutation (e.g., in the promoter region of the MS gene), or it could be due to mutations in a structural gene that affects the k_{cat}. Variation could also be due to variation in the level of vitamin B_{12}, which is a cofactor for MS. In the first two cases variation is genetic, and in the latter case the variation is environmental (e.g., due to a vitamin B_{12} deficiency). Allowing parameters to vary continuously makes it possible to explore a broad range of possibilities (corresponding to a range of alleles with minor effects, or a range of environmental exposures) around the normal or wild type, indicated by the open circles in Fig. 2.9. The shape

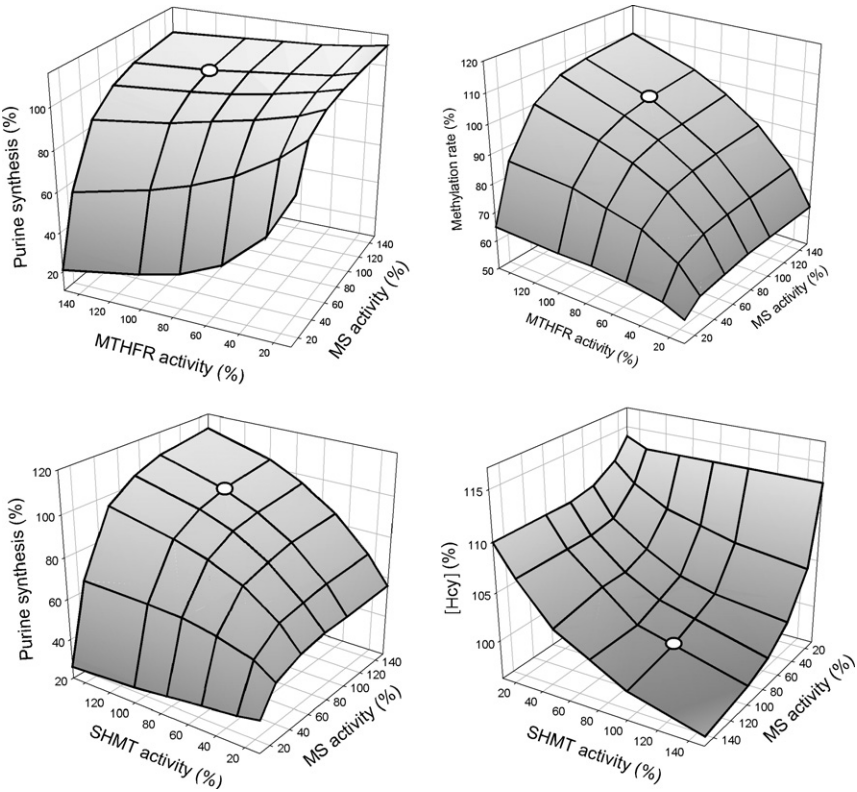

Figure 2.9 Bivariate graphs showing the interaction of various enzymes in FOCM on selected traits (Z axes). X and Y axes show variation in the V_{max} of the respective enzymes. This variation could be due to allelic variation or, in the case of MS, to variation in vitamin B_{12}. The circle shows the location of the normal or wild-type phenotype.

of the interaction relationship is clearly nonlinear, so the effect of variation in MS depends on the exact value of MTHFR activity, and vice versa.

The effects of the interaction of MS and SHMT activity on purine synthesis and homocysteine concentration are shown in Fig. 2.9. These relationships are also nonlinear, as indeed are all relationships between variables within FOCM. In many cases, such as the ones illustrated here, the wild-type values lie on a relatively flat and "horizontal" region of the phenotypic surface. This indicates that the wild type is relatively insensitive to variation in parameter values, because modest variation of the variables or parameter values (x and y axes) has little effect on the phenotype (z axis). As the parameter values move far away from the normal, or wild type, the effect of their variation increases dramatically.

The finding that many wild-type phenotypes lie in regions of the phenotypic surface that are relatively flat and horizontal, implies that the

system is relatively insensitive to the exact values of those parameters. From an evolutionary perspective one would therefore not expect strong selection to maintain those parameter values within close tolerances, because moderate variation has little effect on the phenotype. This observation may help explain why reports on parameter values from different preparations are often inconsistent. Although differences in protocols and experimental errors surely play a role, it is not unreasonable to assume that some of this variation may be real. It is possible that many genes accumulate small-effect mutations in their regulatory region, or their coding region, that would be neutral to selection. Indeed, human genes exhibit abundant single nucleotide polymorphism (SNP) variation. The HapMap Project has uncovered a polymorphic SNP on average every 825 base pairs, and on the average 2 nonsynonymous SNPs per gene (International HapMap Consortium, 2007; McVean *et al.*, 2005). In addition, there is a large amount of regulatory variation in the promoter region of genes that leads to variation in the level of expression (Rockman and Wray, 2002; Yan *et al.*, 2002). A survey of naturally occurring polymorphisms in the promoter regions of 107 human genes showed that 60% caused more than a twofold difference in expression, and 11% caused more than a tenfold difference in expression (Rockman and Wray, 2002). Finally, within a genetically identical population of cells the concentration of a given protein can vary by as much as 30% from cell to cell and from time to time (Elowitz *et al.*, 2002; Sigal *et al.*, 2006).

Thus, there is far more individual genetic variation and individual variation in gene expression than is typically assumed. This, together with the fact that the metabolites and allosteric effectors involved in FOCM vary among individuals and from time to time (e.g., Fig. 2.10), suggests that much of this variation is without significant effect on fitness, and is therefore not under selection, and may therefore explain some of the observed interindividual variability.

VII. Modeling and Simulation have Revealed Novel Homeostatic Mechanisms

FOCM has many functions that must continue to operate normally in face of variation in the demand on specific reactions and variation in the input of metabolites. For instance, the expression levels of TS and DHFR are upregulated more than 100-fold during the S-phase of the cell cycle, when there is an increased demand for nucleotide synthesis (Bjarnason *et al.*, 2001; Obama *et al.*, 2002; Slansky *et al.*, 1993; Wade *et al.*, 1995). At the same time there will be an increased demand for DNA methylation to maintain the correct methylation pattern of the newly synthesized DNA strands. FOCM is also subject to great hourly and daily variation in amino acid input, which varies with meals and nutrition. The amino acids serine

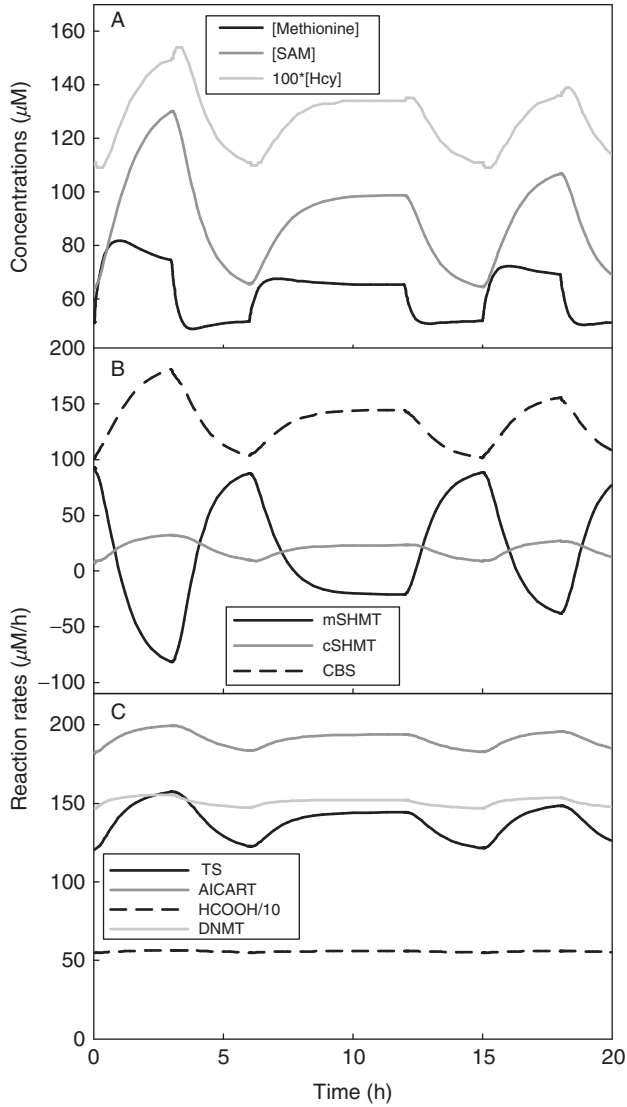

Figure 2.10 Simulated response of selected hepatic FOCM metabolite concentrations and reaction velocities to periodic pulses of amino acid input. (A) Concentration profiles of methionine cycle metabolites. (B) Velocity profiles of the CBS reaction and the mitochondrial and cytosolic SHMT reactions (mSHMT and cSHMT, respectively). (C) Velocity profiles of the DNMT, TS, and AICART reactions and the rate of export of formate (vHCOOH) from the mitochondria. Modified from Nijhout *et al.* (2007b).

and glycine are the primary methyl donors for FOCM, and methionine is both a methyl donor and an essential amino acid linking the folate and methionine cycles. An interesting question is whether and how the stability of critical reactions in the cycle are maintained when there are large localized changes in demand, or large localized changes in input.

Perhaps the best way to illustrate the relative stability of some reactions in the face of variation in inputs is by simulating a day in the life of FOCM. After each meal with protein, the human body experiences a pulse of amino acids that lasts about 3 h. We simulated this by pulsing the four amino acids that serve as inputs for the model (Fig. 2.10). It is evident from the simulations shown in Fig. 2.10 that some variables change dramatically with each meal, while others are almost unaffected. The TS and DHFR reactions are quite stable as are the DNMT rate and the rate of export of formate from the mitochondria. By contrast, the SHMT reactions fluctuate greatly as do the concentrations of SAM and homocysteine.

As discussed above (Section III.D), the stability of formate export from the mitochondria arises from the dynamical interplay between the mitochondrial and cytosolic SHMT reactions, whose magnitude and direction vary with serine and glycine input. The fluctuations in SHMT velocity are a dynamic homeostatic mechanism that dampens the effects of fluctuations in glycine and serine input (Nijhout *et al.*, 2007a).

The methylation of DNA is an important function of FOCM, and it seems reasonable to stabilize these reactions against a variable and often unpredictable input of methyl groups. Simulations with our models show that the DNMT reaction is extraordinarily stable against variation in input, and that this stability arises from two allosteric interactions between the folate cycle and the methionine cycle: SAM inhibits MTHFR and 5mTHF inhibits GNMT (see Fig. 2.11). Wagner *et al.* (1985), and Wagner (1995) suggested that the purpose of these interactions might be to stabilize the rate of DNA methylation. The general idea of how this mechanism works is easy to understand. If the concentration of SAM goes up, then MTHFR is inhibited, which causes the concentration of 5mTHF to fall. When 5mTHF is lower, the inhibition of GNMT is released causing the rate of the GNMT reaction to go up, utilizing the extra SAM and allowing the DNMT rate to remain stable. The reverse scenario explains what happens if SAM goes down.

We experimented with the model shown in Fig. 2.11 by adding and removing the long-range allosteric regulations in various combinations. Figure 2.12 shows how the [SAM]/[SAH] ratio and the DNMT reaction rate vary as a function of methionine input under two scenarios: with all allosteric interaction present, and with all allosteric interactions absent. It is clear that the allosteric interactions stabilize the SAM/SAH ratio and the DNMT reaction rate against variation in methionine input, and that the effect is most pronounced at low methionine input. This is because under an optimal supply of methionine the DNMT reaction runs close to saturation,

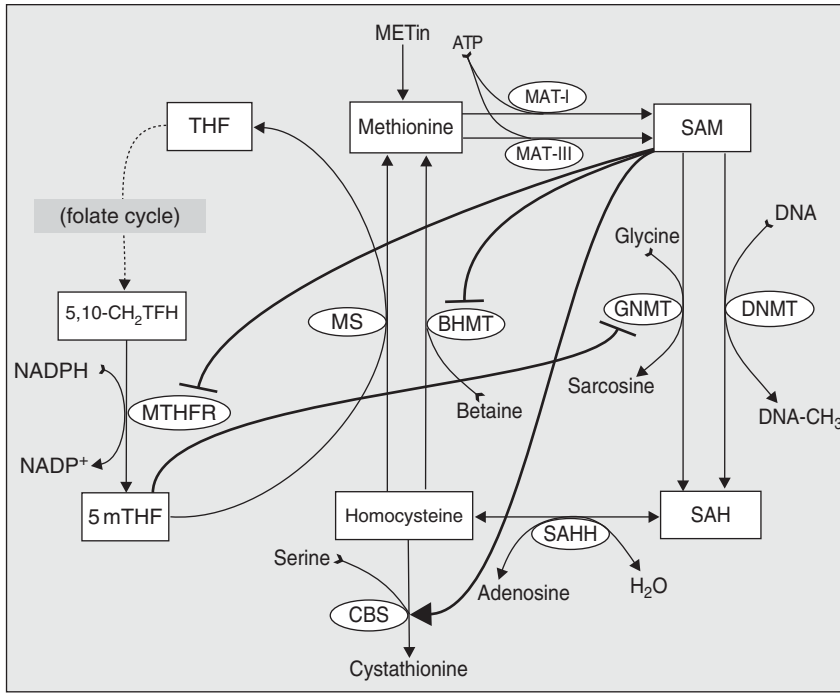

Figure 2.11 Model of the allosteric regulatory interactions within the methionine cycle and between the folate and methionine cycles used to study the stability of the DNMT reaction against fluctuations in methionine input. Thick lines show the allosteric interactions of SAM and 5mTHF. Arrow indicates activation and bars indicate inhibition. Redrawn from Nijhout *et al.* (2006).

so the main benefit of these regulations appears to be to protect the DNMT reaction against periods of protein starvation.

Finally, as noted above, the expression of TS and DHFR vary a 100-fold or more with various stages of the cell cycle, and we have shown, by simulation, that this variation has little or no effect on the reaction velocities and metabolite concentrations elsewhere in the folate and methionine cycles (Nijhout *et al.*, 2004). An implication of this funding is that FOCM should be relatively insensitive to inhibition of TS and DHFR by chemotherapeutic drugs such as methotrexate (which inhibits DHFR) and 5-fluoro-uracil (which inhibits TS). This is indeed the case. When the V_{max} of DHFR is lowered (corresponding, e.g., to treatment with methotrexate) the velocity of the DHFR reaction remains virtually constant until there is almost no free enzyme left. The reason for this remarkable stability of the DHFR reaction is that the normal concentration of its substrate, DHF, is exceptionally low, typically 0.02 μM out of a total folate pool of 20 μM. Thus, the concentration of DHF can rise more than a 100-fold

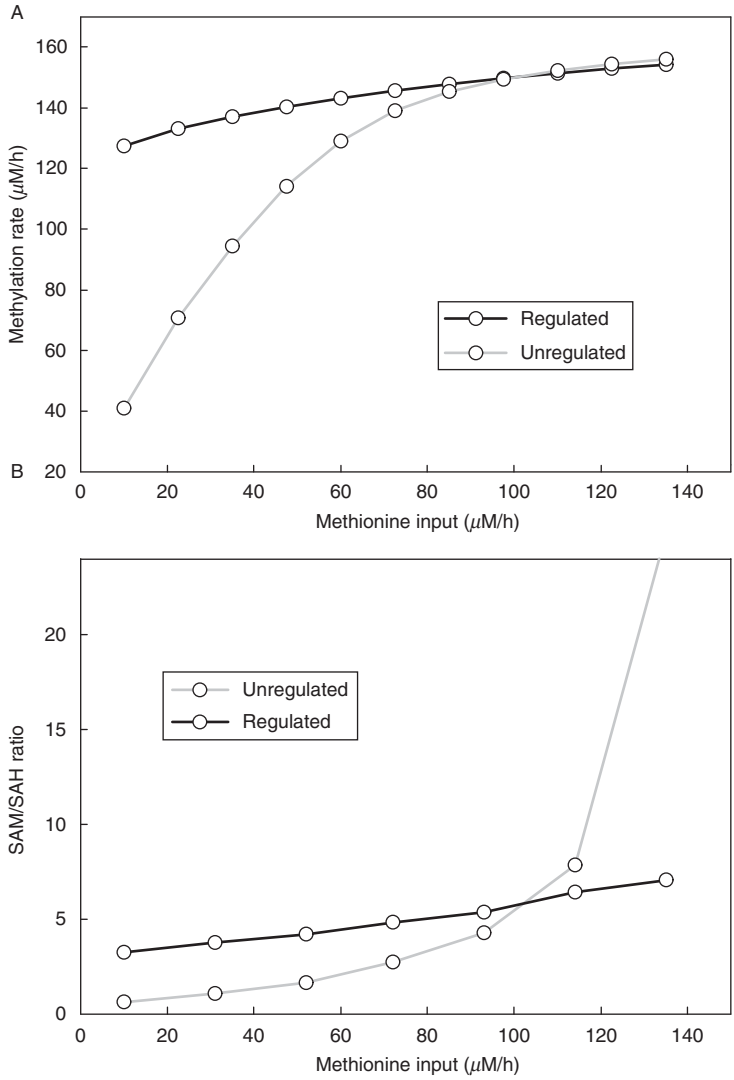

Figure 2.12 Effect of allosteric regulation by SAM and 5mTHF on the response of (A) the [SAM]/[SAH] ratio and (B) the DNMT reaction to variation in methionine input. Solid line shows the response when all allosteric regulations are in place, and the dotted line shows the response without allosteric regulation. Allosteric regulation stabilizes both the [SAM]/[SAH] ration and DNMT reaction at low methionine input, and thus may be an adaptation to protein starvation.

(and drive the DHFR reaction by substrate accumulation) without substantially depleting the folate pool and disrupting the reaction rates elsewhere in FOCM. The inhibition of DHFR by methotrexate in effect creates a DHF

trap. The rate at which this DHF trap develops is determined by the rate of the TS reaction. In a rapidly dividing cancer cell, where TS is highly up-regulated, the DHF trap will develop rapidly, whereas in a non-cancerous, non-dividing cell it will develop very slowly if at all.

VIII. Steady States and Fluctuations

The mathematical models allow us to calculate how long it takes for FOCM to return to steady state after a perturbation. The interlocking cycles of FOCM are complex and different reactions return to steady state at different rates. An example is shown in Fig. 2.7 where we show the simulated response to fasting. It takes 6–10 h for some reactions to go to steady state while others take more than 2 days. Given a normal pattern of eating, these findings imply that FOCM is never at steady state (Nijhout *et al.*, 2007b). Indeed many reaction rates and metabolite concentrations are likely to always be far from steady state (Fig. 2.10).

This calls into question the utility of standard metabolic control analysis to understand the operation of this system. In metabolic control analysis one typically lets the system come to steady state, then perturbs it by changing one parameter by a small amount, and lets the system come to the new steady state (Fell, 1992). The fractional change in the reaction velocities and metabolite concentrations at this new steady state is then taken to be a measure of the sensitivity of each component of the system to the parameter that was changed. This method is used to deduce how control is distributed among the reactions of a system, and the relative control any given enzyme has over the operation of the system. Metabolic control analysis is, in effect, a sensitivity analysis preformed by perturbing the steady state. When a system normally operates far from steady state, and its reaction velocities and metabolite concentrations are continually changing, a steady-state sensitivity analysis is not a useful way of obtaining insight into the operation of the system.

Instead, it is more natural to see how the system responds to large scale fluctuations. We have been using such fluctuations in different ways. First, we have been using fluctuations to make quantitative statements about the effects of particular homeostatic regulatory mechanisms. For example, in Nijhout *et al.* (2006) we added to the normal methionine input (100 μM/h) a continuous stochastic fluctuation with standard deviation 30 μM/h. The standard deviation in the velocity of the DNA methylation reaction was exceptionally small, because of the long-range allosteric interactions discussed above. We then removed allosteric interactions one by one to see which ones and which combinations had the greatest effects. When all four are removed, the standard deviation of the velocity of the DNMT reaction

goes up by a large factor. We have also used such fluctuation analysis to show that it is the unusual kinetics of MAT-I and MAT-III that stabilizes the methionine concentration at the expense of large fluctuations in SAM (unpublished). In Nijhout *et al.* (2007), we applied stochastic fluctuations to the serine and glycine inputs and showed that the production of formate by the mitochondria remains remarkably stable. This stability is caused by the parallel SHMT reactions in the cytosol and the mitochondria that make glycine from serine and vice versa.

Secondly, we often use external stochastic fluctuations as a probe of system behavior. It is very interesting to fluctuate an input or a $V_{\max}$ (corresponding to gene up- and downregulation) and then observe which concentrations and velocities fluctuate a lot, a moderate amount, or hardly at all. Our experience is that when a concentration or velocity hardly fluctuates at all, there is usually a good biological reason why this is so. We can then take the system apart to discover the mechanisms that cause the homeostatic behavior. Usually, some other concentrations and velocities change a lot so that the homeostatic ones can remain stable.

Finally, we have been conducting a mathematical analysis of the way in which general fluctuations propagate through biochemical networks. In Anderson *et al.* (2007), we showed that the variances of reactions velocities are always strictly decreasing down linear chains. The biological significance of this result is that if it is important to stabilize the output of a chain of biochemical reactions against fluctuations in the input, then the chain should be long. It was also shown that side reaction systems and feedback loops decrease the variations of the velocities in downstream reactions. In Anderson and Mattingly (2008), many of these results are proven in the case of Michaelis-Menten chains. Efforts are underway to prove how more complicated network geometries and different kinds of kinetics affect the ways in which fluctuations propagate.

Finally, we note that metabolic networks do not arise fully formed. They evolve over time by the addition and elimination of reactions and by changes in the kinetics of existing reactions. In evolutionary biology, it is typically assumed that natural selection acts to maximize flux through a pathway (e.g., Hartl *et al.*, 1985; Wagner, 2005), in effect making reactions more "efficient" in some way. But if a system normally experiences continual and large fluctuations of input, and continuous and large changes in the demand for many different synthetic reactions, then a more likely target for natural selection would be those reactions or connections that stabilize certain parts of the system against the effects of those changes. That is, evolution would not necessarily favor faster and more efficient pathways, but rather would favor pathways that operate stably and reliably under variation. Eating imposes enormous hourly and daily fluctuations, as well as unpredictable long-term deficiencies in specific nutrients, and normal daily and seasonal activities impose large variation in demand. This is true of

FOCM, and it must be true of most if not all of metabolism. The key regulatory features of metabolic systems are thus those that stabilize function, and those that prevent local perturbations from propagating through the system. As is the case in FOCM, these regulatory mechanisms are not the emergent properties of large networks, but are evolved adaptations for specific functions.

IX. Conclusions

FOCM is one of the best studied metabolic systems: all or almost all enzymes and metabolites in the system are known, as is the structure of the reaction network. This network is complex and consists of several intersecting cycles and a large number of complex allosteric regulatory interactions between metabolites and enzymes. The reactions in this system are nonlinear, which makes it exceptionally difficult to deduce the properties of the overall system, the way it is regulated, and the effects of mutations and nutrient and vitamin deficiencies from the connectivity diagram alone.

The most direct way to understand the function of different parts of a complex system like FOCM is through computer simulation with a mathematical model. Because FOCM has been so well studied, it has been possible to construct models that accurately simulate metabolite pools and reaction velocities, as well as the effects of mutations and vitamin deficiencies on markers like homocysteine, TS and methylation capacities.

A mathematical model is an experimental tool that can be used as a complement to laboratory experimentation or clinical investigation to do pilot experiments and test hypotheses quickly and inexpensively. When a new interaction is discovered, or suspected, it can be incorporated into a preexisting model to determine its effect. We expect that our mathematical models will evolve in three ways: first by progressive improvement of the accuracy of the existing models by incorporating details like polyglutamation, substrate channeling, and compartmentalization; second, by extending the models to include other related aspects of metabolism, like insulin signaling; third, by developing additional tissue-specific models, for instance, for the brain and transport across the blood–brain barrier, and by linking models for multiple organ systems together through the circulatory system.

One important purpose of studying FOCM is to understand the relationship between genetic and environmental variables and disease outcomes. There are two large steps necessary for this understanding. First, one needs to understand how genetic and the environmental perturbations affect the system behavior of FOCM. Second, one needs to understand how the system changes in FOCM lead to the various disease states. Both are very difficult questions. Most of our work outlined above has been

dedicated to understanding the regulatory system properties of FOCM and how the behavior of FOCM changes in the presence of genetic polymorphisms and changes in environmental input. It remains a formidable challenge to understand the pathway by which inadequacies or malfunctions of the processes regulated by FOCM contribute to the development of such diverse diseases as colon cancer, psychiatric disorders, cardiovascular disease, and neural tube defects.

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