Metabolic Engineering from a Cybernetic Perspective: Aspartate Family of Amino Acids

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Using the modular cybernetic framework developed by Varner and Ramkrishna (Varner and Ramkrishna; 1998a, b) a cybernetic model is formulated that describes the time evolution of the aspartate family of amino acids in Corynebacterium lactofermentum ATCC 21799. The network model formulation is employed in the role of a diagnostic tool for the overproduction of threonine. More precisely, having determined a parameter set that describes the time evolution of a base strain (lysine producer), the model predicted response to genetic perturbations, designed to enhance the level of threonine, are simulated using an appropriately modified cybernetic model and compared with the experimental results of Stephanopoulos and Sinskey (Colon et al., 1995a, Appl. Environ. Microbiol. 61, 74–78) for identical genetic perturbations. It is found that the model predicted response to enzymatic over-expression in the aspartate pathway agrees, for the most part, with experimental observations within the experimental error bounds. This result lends credence to the hypothesis that cybernetic models can be employed to predict the local response of a metabolic network to genetic perturbation, thereby, affording cognizance of the potential pitfalls of a particular genetic alteration strategy a priori. © 1999 Academic Press

Key Words: cybernetic models; metabolic engineering; amino acid production.

I. INTRODUCTION

Millions of years of selective pressure have forced microorganisms to be strongly adaptive and reactive to environmental and genetic perturbation, so as to ensure survival. This trait follows from the unique ability to modulate physiological function via regulatory signals emanating from elaborate control architectures that have been programmed by evolution to meet new and diverse nutritional challenges. Thus, it is not surprising, that metabolic networks are often seen to “resist” alteration through adjustments of functionality by way of shifts in protein expression which in turn impacts enzymatic activity. Indeed, there exists a seemingly innumerable assemblage of experimental developments with the shared objective of exploring system response to altered levels of enzymatic machinery in the hope of mapping system regulatory action or obtaining organisms with desired features (Brown, 1997). Clearly, however, given the ill-defined, intricately complex nature of metabolic networks, such a carefree approach to metabolic design is in reality only slightly better than a hit or miss proposition. This is the case because the very same control mechanisms that confer behavioral robustness and adaptability to microorganisms frequently frustrate metabolic engineering efforts (defined as the useful modification of physiological function via recombinant DNA technology (Bailey, 1991; Stephanopoulos and Vallino, 1991)) by proactively resisting attempts to redistribute metabolic flux.

With that said, given the level of intellectual involvement currently invested in metabolic engineering efforts, it is true that progress has been made. However, no one would argue that the rational design of metabolic pathways has progressed to the point of being a functional engineering discipline (Bailey, 1991). The biological tools are in place for relatively easy genetic manipulation of metabolic network functionality. Thus, this step in the design process can no longer be considered as the limiting factor, which arguably was not the case in the past. Rather, currently, the rigorous design guidance that mathematical analysis can provide is sorely lacking. Mathematical tools such as Metabolic Control Analysis (MCA) developed by Kacer and Burns (Kacer and Burns, 1973) and Biochemical Systems Theory (BST) developed by Savageau (Savageau, 1976) attempt to describe the control of metabolic networks through the use of sensitivity coefficients. The results of attempts to a priori rationally design metabolic networks using these tools have met with limited success with some notable exceptions (Hatzimanikatis, Floudas and Bailey,
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The conceptual extension of classic cybernetic doctrine to metabolic engineering has been presented in detail 
elsewhere (Varner and Ramkrishna, 1998a, Varner and Ramkrishna, 1998b) so we dispense with a formal first 

principles discussion, instead we present only a brief review of the salient conceptual features. Thus, our primary 
focus is not a reiteration of the basis of the extension, rather, it is to evaluate the potential of the framework by analyzing a realistic case study. In particular, we consider the 
overproduction of members of the aspartate family of amino acids. In Corynebacterium spp., lysine, methionine, threonine, and isoleucine derive part or all of their carbon from aspartate. The pathway flux that leads to the forma-
tion of these intermediates is controlled by regulatory from aspartate. The pathway flux that leads to the forma-
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metabolic control analysis is not a modeling framework, rather, it is a set of postulates (following simply from the 
chain rule of differentiation) that allow the systematic com-
putation of localized network sensitivities to single pertur-
bations in the environmental or network parameters. Thus, its predictive capability is limited by the quality and source of the information that is input into the analysis structure.

Surely, an explicit dependence upon experimental input negates any type of a priori predictive potential because the results of the experiment are required for the analysis. If the analysis input stems from a kinetic model of the network, the claim that the sensitivity coefficients reflect the input of metabolic control mechanisms is suspect because traditional kinetic models often lack a closed description of the regulatory component (in the best case a representation of metabolite level regulation may be afforded via the kinetics expressions.) In this instance, the sensitivity coefficients do not describe regulatory effects, rather, they reflect the influence upon system behavior of kinetic competition within the network. Biochemical systems theory does contain a modeling aspect, and as such, theoretically it possesses the potential of being predictive in scope. However, it also depends upon sensitivity arguments to develop a conception of network regulation. The explicit dependence upon sensitivity coefficients, which in general are valid only locally, limits the predictive scope of this framework as well. Perhaps a more fundamental reason for the limited success of the mathematical tools discussed above is the founding belief that metabolic networks are “static” reaction systems. In other words, often enzyme level as well as activity are treated as static parameters (influencing \( V_{\text{max}} \) in some ad hoc manner), an assumption which is clearly questionable for cases in which the makeup of the metabolic machinery is being significantly modulated. On the other hand, very detailed representations of metabolic regulation at the gen-
etic level and moreover, its impact upon protein expression, such as that provided by the pioneering work of Lee and Bailey (Lee and Bailey, 1984) while being an ideal alter-

The application of the cybernetic framework, developed by Ramkrishna and co-workers, for the description and analysis of metabolic networks is a new approach and may offer a distinct advantage over traditional methodologies. The cybernetic framework is the only modeling framework in existence that affords a systematic representation of the influence of metabolic regulation, both the control of enzyme activity as well as at the genetic level. The frame-
work hypothesizes that metabolic systems have evolved optimal goal oriented regulatory “programs” as a result of evolutionary pressures to cope with environmental and/or genetic perturbation. The optimality hypothesis implies the system directs the synthesis and activity of network enzymes such that a nutritional objective is achieved in an optimal manner. The inclusion of a goal oriented regulatory strategy
gives the cybernetic description of a metabolic network the key feature of regulatory reactivity, an element that is mis-
sing from all other contemporary metabolic network analysis and modeling frameworks. In other words, the feature that a cybernetic realization of a metabolic net-
work offers, that is absent from all other contemporary approaches, potentially, is the ability to determine the manner in which a metabolic network redirects enzymatic expression and activity in the face of environmental pertur-
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to describe the local regulatory reactivity of a metabolic network and contributes, in a limited sense, to the proof of the conceptual basis of the extension of the cybernetic framework to metabolic engineering applications and especially recombinant systems as put forth by the postulates presented in Varner and Ramkrishna (Varner and Ramkrishna, 1998).

1.1. Scope of the Investigation

Using the modular concept introduced in Varner and Ramkrishna (Varner and Ramkrishna, 1998a) a cybernetic model is constructed which describes the time evolution of the aspartate family of amino acids. This model system is used to investigate genetic alterations designed to enhance the level of amino acids present within the network. In particular, we compare the model predicted response to genetic perturbations with those obtained experimentally by Stephanopoulos and Sinskey (Colón et al., 1995a, b; Jetten et al., 1995) and co-workers for the overproduction of lysine and threonine.

We begin the development by outlining the process equations and kinetics that constitute the model framework shown in Fig. 1. Once this is accomplished, we turn our attention to making the model system complete by formulating the appropriate cybernetic variables that modify the various catalytic rates and the rates of enzyme synthesis.

2. MATERIALS AND METHODS

All models discussed within the development were constructed and simulated within the Simulink environment of Matlab. The model equations were numerically evaluated using the ODE15s routine of Matlab. Unless otherwise noted, the simulation parameter set was that shown in Table 1.

### TABLE 1
Sample Parameter Set Aspartate Family Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu^{\text{ass}}$</td>
<td>1.25 (g/gDW \cdot h)</td>
</tr>
<tr>
<td>$\mu^{\text{ass}}_b$</td>
<td>0.4 (g/gDW \cdot h)</td>
</tr>
<tr>
<td>$\mu^{\text{ass}}_i$</td>
<td>0.25 (g/gDW \cdot h)</td>
</tr>
<tr>
<td>$\mu^{\text{ass}}_k$</td>
<td>0.05 (g/gDW \cdot h)</td>
</tr>
<tr>
<td>$\mu^{\text{ass}}_s$</td>
<td>0.25 (g/gDW \cdot h)</td>
</tr>
<tr>
<td>$K_{D}$</td>
<td>0.01 (g/gDW)</td>
</tr>
<tr>
<td>$K_{i}^{\text{as}}$</td>
<td>0.001 (g/gDW)</td>
</tr>
<tr>
<td>$K_{s}^{\text{as}}$</td>
<td>0.01 (g/gDW)</td>
</tr>
<tr>
<td>$K_{b}$</td>
<td>0.01 (g/gDW)</td>
</tr>
<tr>
<td>$K_{i}^{\text{as}}$</td>
<td>0.25 (g/gDW)</td>
</tr>
<tr>
<td>$Y_{x,S}$</td>
<td>0.0219 (gDW/g)</td>
</tr>
</tbody>
</table>

3. PROCESS EQUATIONS AND KINETICS

In this section we outline the process equations and kinetics which constitute the model framework shown in Fig. 1. Once this is accomplished, we turn our attention to making the model system complete by formulating the appropriate cybernetic variables that modify the various catalytic rates and the rates of enzyme synthesis.
The intermediate $p_0$ is supplied from upper pathway metabolism at the rate $R_0$ and is consumed via the key enzyme $e_0$ at the specific rate

$$
r_0 = \mu_0^{\text{max}} \left( \frac{e_0}{e_0^{\text{max}}} \right) \frac{p_0}{K_0 + p_0},
$$

(3.1)

where $\mu_0^{\text{max}}$, $K_0$ are the rate an saturation constants governing the consumption of $p_0$ and $e_0^{\text{max}}$ denotes the maximum level of key enzyme $e_0$. The key enzyme $e_0$ is assumed to be induced by the presence of $p_0$ at the specific rate

$$
r_0 = \alpha_0 \frac{p_0}{K_{\alpha_0} + p_0},
$$

(3.2)

where $\alpha_0$, $K_{\alpha_0}$ denote the rate and saturation constants governing the synthesis of $e_0$.

The intermediate $p_1$ is consumed to produce the metabolite $p_2$ via the key enzyme $e_1$ at the specific rate

$$
r_1 = \mu_1^{\text{max}} \left( \frac{e_1}{e_1^{\text{max}}} \right) \frac{p_1}{K_1 + p_1},
$$

(3.3)

where $\mu_1^{\text{max}}$, $K_1$ are the rate and saturation constants governing the synthesis of $p_2$ and $e_1^{\text{max}}$ denotes the maximum level of key enzyme $e_1$. The key enzyme $e_1$ is assumed to be induced by $p_1$ and is expressed at the specific rate

$$
r_1 = \alpha_1 \frac{p_1}{K_{\alpha_1} + p_1},
$$

(3.4)

where $\alpha_1$, $K_{\alpha_1}$ are the rate and saturation constants governing the expression of $e_1$.

The branch point precursor metabolite $p_2$ can be consumed to produce metabolite $p_3^a$ or $p_3^b$. The formation of $p_3^a$ catalyzed by key enzyme $e_3^a$ proceeds according to the specific rate

$$
r_3^a = \mu_3^{a, \text{max}} \left( \frac{e_3^a}{e_3^{a, \text{max}}} \right) \frac{p_3^a}{K_3^a + p_3^a},
$$

(3.5)

where $\mu_3^{a, \text{max}}$, $K_3^a$ are the rate and saturation constants governing the formation of $p_3^a$ and $e_3^{a, \text{max}}$ denotes the maximum level of key enzyme $e_3^a$. The key enzyme $e_3^a$ is assumed to be induced by the intermediate $p_2$ and is expressed according to the specific rate

$$
r_3^a = \alpha_3^a \frac{p_2}{K_{\alpha_3^a} + p_2},
$$

(3.6)

where $\alpha_3^a$, $K_{\alpha_3^a}$ denote the rate and saturation constants governing $e_3^a$ expression. The formation of $p_3^b$ is catalyzed by key enzyme $e_3^b$ and proceeds according to the specific rate

$$
r_3^b = \mu_3^{b, \text{max}} \left( \frac{e_3^b}{e_3^{b, \text{max}}} \right) \frac{p_3^b}{K_3^b + p_3^b},
$$

(3.7)

where $\mu_3^{b, \text{max}}$, $K_3^b$ denote the rate and saturation constants governing the rate of formation of $p_3^b$ and $e_3^{b, \text{max}}$ denotes the maximum level of key enzyme $e_3^b$. The key enzyme $e_3^b$ is assumed to be induced by the presence of $p_2$ and is expressed at the specific rate

$$
r_3^b = \alpha_3^b \frac{p_2}{K_{\alpha_3^b} + p_2},
$$

(3.8)

where $\alpha_3^b$, $K_{\alpha_3^b}$ denote the rate and saturation constants governing the expression of $e_3^b$.

The branch intermediate $p_3^a$ is assumed to be consumed for lower pathway metabolic activity at the specific rate

$$
R_{\mu_3} = \mu_3^{a, \text{max}} \frac{p_3^a}{K_3^a + p_3^a},
$$

(3.9)

where $\mu_3^{a, \text{max}}$, $K_3^a$ denote the rate and saturation constants governing the utilization of $p_3^a$. Note because the structure of the lower metabolic pathway is not included in the model framework, no regulation can be assigned to the key enzyme which mediates the utilization of $p_3^a$. However, this enzyme level must be responsive to changes in the nutritional state of the microorganism and is not considered a constant. We consider the representation of the interaction of the lower pathway metabolic pathway and the nutritional state of the organism with the aspartate pathway in the metabolic regulation section. The branch metabolite $p_3^b$ can be consumed to produce both $p_3^{ab}$ and $p_4^{bb}$. The formation of $p_3^{ab}$ is catalyzed by $e_3^{ab}$ and proceeds according to the specific rate

$$
r_3^{ab} = \mu_3^{a, \text{max}} \left( \frac{e_3^{ab}}{e_3^{a, \text{max}}} \right) \frac{p_3^{ab}}{K_3^{ab} + p_3^{ab}},
$$

(3.10)

where $\mu_3^{a, \text{max}}$, $K_3^{ab}$ denote the rate and saturation constants governing the formation of $p_3^{ab}$ and $e_3^{a, \text{max}}$ denotes the maximum level of key enzyme $e_3^{ab}$. The key enzyme $e_3^{ab}$ is assumed to be induced by $p_3^a$ and is expressed at the specific rate

$$
r_3^{ab} = \alpha_3^{ab} \frac{p_3^a}{K_{\alpha_3^{ab}} + p_3^a},
$$

(3.11)
where $\alpha^{\text{bb}}_5$, $K_5^b$ denote the rate and saturation constants governing enzyme synthesis. The formation of $p_4^{\text{bb}}$ is catalyzed by the key enzyme $e_3^b$ and proceeds according to the specific rate

$$
 r_3^{\text{bb}} = \mu_3^{\text{bb,max}} \left( \frac{e_3^{\text{bb}}}{e_3^{\text{bb,max}}} \right) \frac{p_4^{\text{bb}}}{K_4^b + p_4^{\text{bb}}}, \quad (3.12)
$$

where $\mu_3^{\text{bb,max}}$, $K_4^b$ denote the rate and saturation constants governing the formation $p_4^{\text{bb}}$ and $e_3^{\text{bb,max}}$ denotes the maximum level of key enzyme $e_3^b$. The key enzyme $e_3^b$ is assumed to be induced by $p_3^b$ and is expressed according to the specific rate

$$
 r_3^b = \alpha^{b}_3 \frac{p_3^b}{K_3^b + p_3^b}, \quad (3.13)
$$

where $\alpha^b_3$, $K_3^b$ denote the rate and saturation constants governing the expression of $e_3^b$.

The intermediate $p_4^{\text{ba}}$ is assumed to be utilized by downstream metabolic processes according to the specific rate

$$
 R_4^{\text{ba}} = \mu_4^{\text{ba}} \left( \frac{p_4^{\text{ba}}}{e_4^{\text{ba,max}}} \right), \quad (3.14)
$$

where $\mu_4^{\text{ba}}$, $K_4^{\text{ba}}$ denote the rate and saturation constants that govern the utilization of $p_4^{\text{ba}}$. Following the $p_3^b$ discussion, the dynamics of the key enzyme that catalyzes downstream utilization of $p_4^{\text{ba}}$ are considered in the metabolic regulation section. The intermediate $p_4^{\text{ba}}$ is consumed to form $p_5$ via key enzyme $e_5^{\text{ba}}$ according to the specific rate

$$
 r_4 = p_4^{\text{bb}} = \mu_4^{\text{ba,max}} \left( \frac{e_4^{\text{ba}}}{e_4^{\text{ba,max}}} \right) \frac{p_4^{\text{ba}}}{K_4^{\text{ba}} + p_4^{\text{ba}}}, \quad (3.15)
$$

where $\mu_4^{\text{ba,max}}$, $K_4^{\text{ba}}$ denote the rate and saturation constants and $e_4^{\text{ba,max}}$ denotes the maximum level of key enzyme $e_4^{\text{ba}}$. The key enzyme $e_4^{\text{ba}}$ is assumed to be induced by the presence of $p_4^{\text{ba}}$ and is expressed at the specific rate

$$
 r_4^b = r_4^{\text{bb}} = \alpha^{\text{bb}}_4 \frac{p_4^{\text{bb}}}{K_4^b + p_4^{\text{bb}}}, \quad (3.16)
$$

where $\alpha^{\text{bb}}_4$, $K_4^b$ denote the rate and saturation constants governing the expression of $e_4^{\text{bb}}$.

The metabolite $p_5$ is consumed to produce $p_6$ via the key enzyme $e_5$ according to the specific rate

$$
 r_5 = \mu_5^{\text{max}} \left( \frac{e_5}{e_5^{\text{max}}} \right) \frac{p_5}{K_5 + p_5}, \quad (3.17)
$$

where $\mu_5^{\text{max}}$, $K_5$ denote the rate and saturation constants which govern $p_5$ formation and $e_5^{\text{max}}$ denotes the maximum level of key enzyme $e_5$. The key enzyme $e_5$ is assumed to be induced by $p_5$ and is expressed at the specific rate

$$
 r_5 = \alpha_5 \frac{p_5}{K_\alpha_5 + p_5}, \quad (3.18)
$$

where $\alpha_5$, $K_\alpha_5$ denote the rate and saturation constants governing the synthesis of $e_5$.

The intermediate $p_6$ is consumed to form $p_7$ via the key enzyme $e_7$ according to the specific rate

$$
 r_6 = \mu_6^{\text{max}} \left( \frac{e_6}{e_6^{\text{max}}} \right) \frac{p_6}{K_6 + p_6}, \quad (3.19)
$$

where $\mu_6^{\text{max}}$, $K_6$ denote the rate and saturation constants governing the formation of $p_7$ and $e_6^{\text{max}}$ denotes the maximum level of key enzyme $e_6$. The key enzyme $e_6$ is assumed to be induced by $p_6$ and is expressed at the specific rate

$$
 r_6 = \alpha_6 \frac{p_6}{K_\alpha_6 + p_6}, \quad (3.20)
$$

where $\alpha_6$, $K_\alpha_6$ denote the rate and saturation constants which govern the synthesis of $e_6$.

The intermediate $p_7$ is consumed to form $p_8$ via the key enzyme $e_7$ according to the specific rate

$$
 r_7 = \mu_7^{\text{max}} \left( \frac{e_7}{e_7^{\text{max}}} \right) \frac{p_7}{K_7 + p_7}, \quad (3.21)
$$

where $\mu_7^{\text{max}}$, $K_7$ denote the rate and saturation constants governing the formation of $p_7$ and $e_7^{\text{max}}$ denotes the maximum level of key enzyme $e_7$. The key enzyme $e_7$ is assumed to be induced by $p_7$ and is expressed at the specific rate

$$
 r_7 = \alpha_7 \frac{p_7}{K_\alpha_7 + p_7}, \quad (3.22)
$$

where $\alpha_7$, $K_\alpha_7$ denote the rate and saturation constants which govern the synthesis of $e_7$.

The intermediate $p_8$ is assumed to be utilized by downstream metabolic processes according to the specific rate

$$
 R_8 = \mu_8^{\text{max}} \left( \frac{p_8}{K_8 + p_8} \right), \quad (3.23)
$$
where \( \mu_{\text{max}}^r \), \( K_r \) denote the rate and saturation constants which govern the utilization of \( p_g \). As per the previous discussion, the dynamics of the key enzyme which catalyzes \( p_g \) utilization are discussed in the metabolic regulation section.

We assume that the \( j \)th metabolic intermediate can diffuse from the cell at the specific rate

\[
r_{d,j} = k_{d,j} (p_j - \varphi p_j), \quad j = 3a, 3b, 4ba, 4bb, 5, 8,
\]

where \( p_j \) denotes the abiotic phase level of \( p_j \) and \( \varphi \) denotes the specific cellular volume.

The model processes shown in Fig. 1 and discussed above are specific, i.e., are written with respect to the biotic phase. Accordingly, a representation of the growth process must accompany the description of the metabolic network. Because we seek to maintain generality, we postulate a simple structured cybernetic growth model to describe the time evolution of the biotic phase and macroscopic variables such as growth substrate and biomass level. The growth model framework is shown in Fig. 2. In particular, we postulate the growth substrate \( S_g \) which in this case is glucose, is assimilated and converted to the lumped growth precursor pool \( p_g \) via the key enzyme \( e_{T,g} \) at the specific rate

\[
r_{T,g} = \mu_{\text{max}}^r \frac{e_{T,g}}{K_{T,g} + e_{T,g}} \frac{S_g}{S_g + e_{T,g}},
\]

where \( \mu_{\text{max}}^r \), \( K_{T,g} \) denote the maximum rate and saturation constants that govern the formation of the growth precursor pool \( p_g \) and \( e_{\text{max}} \) denotes the maximum specific level of the key transport enzyme \( e_{T,g} \). The key enzyme \( e_{T,g} \) is assumed to be induced by the growth substrate \( S_g \) and is expressed at the specific rate

\[
r_{e_{T,g}} = \alpha_{T,g} \frac{S_g}{K_{T,g} + S_g},
\]

where \( \alpha_{T,g} \), \( K_{T,g} \) denote the rate and saturation constant governing the synthesis of \( e_{T,g} \) once present within the cell, the intermediate \( p_g \) can be consumed to produce biomass via the lumped key enzyme \( e_g \) at the specific rate

\[
r_{g} = \mu_{\text{max}}^g \frac{e_g}{K_{T,g} + e_{T,g}} \frac{p_g}{p_g + e_g},
\]

where \( \mu_{\text{max}}^g \), \( K_g \) denote the maximum specific rate of \( p_g \) consumption and the saturation constant governing the rate of biomass formation, respectively, and \( e_{\text{max}} \) denotes the maximum specific level of key enzyme \( e_g \). The key enzyme \( e_g \) is assumed to be induced by \( p_g \) and is expressed at the specific rate

\[
r_{e_g} = \alpha_{g} \frac{p_g}{K_{g} + p_g},
\]

where \( \alpha_{g} \), \( K_{g} \) denote the rate and saturation constants which govern the expression of \( e_g \).

When considering the overexpression of system enzymes, we assume the use of both artificial and native promoters. The artificial promoter (in this case) does not allow transcription to occur unless a particular chemical inducing agent is present within the biotic phase. This is equivalent to, for example, the use of a tac or lac promoter that is sensitive to levels of isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG). We denote the abiotic phase level of the \( j \)th promoter inducer by \( I_j \). The inducer is assumed to be assimilated by the carrier protein \( e_T \) at the specific rate

\[
r_{e_T} = \alpha_{e_T} \frac{I_j}{K_{e,T} + I_j},
\]

where \( \alpha_{e_T} \), \( K_{e,T} \) denote the rate and saturation constants governing the assimilation of \( I_j \) and \( e_{\text{max}}^{e_T} \) denotes the maximum level key enzyme \( e_{T} \). The lumped transport enzyme \( e_T \) is assumed to be induced by the presence of \( S_e \) and is expressed at the specific rate

\[
r_{e_T} = \alpha_{T,g} \frac{S_e}{K_{e,T} + S_e},
\]

where \( \alpha_{T,g} \), \( K_{g} \) denote the rate and saturation constants governing the synthesis of \( e_{T} \). Note the induction by the growth substrate \( S_e \) implies inducer transport is a consequence of the presence of the growth substrate.

### 3.1. Metabolic Regulation: Network

In what follows the modular approach for the formulation of the cybernetic variables governing the synthesis and activity of enzymatic machinery is summarized. Additionally, we briefly discuss the theoretical basis for the extension of the cybernetic framework to genetically altered
3.1.1. Review: Modular Approach

At the core of the concept is the postulate that metabolic networks are segmented, i.e., they are composed of a collection of interacting elementary pieces. This understanding implies that networks can be decomposed into a set of interacting elementary pathways, akin to, for example the pieces of a jigsaw puzzle (where interaction between elementary pathways can be via material flux or regulatory communication.) The converse of this postulate implies that given a “library” of elementary pathways, it is possible to construct the topological realization of an arbitrary metabolic network by simply assembling the members of this library in an appropriate fashion. The regulatory portrait of the system then follows from the method of assembly. This is true because each of the elementary pathways is a discrete cybernetic subunit, in other words, we postulate that every elementary pathway in the “library” has a cybernetic resource allocation problem associated with it. Thus, each elementary pathway has a particular regulatory “flavor” stemming from its isolated objective and it is the interplay between these different modes of regulation that determine the complete regulatory character of the network. Our elementary pathway “library” consists, in this case, of generalizations of the elementary building blocks proposed by Straight and Ramkrishna (Straight and Ramkrishna, 1994).

The second founding postulate then goes as to how the individual regulatory influences (formally termed elementary regulatory signals) are “fused” into a “complete” regulatory signal, where “fusion” consists of not only the amalgamation of elementary regulatory flavors, but the manner in which seemingly unconnected network elements communicate. To accomplish the systematic integration of all signals into a complete regulatory signal, we postulate that metabolic regulation broadly consists of three interacting layers, termed elementary, local and global. The elementary level, as alluded to previously, is that influence directly associated with the individual elementary pathways in our “library.” The local level then follows from the interaction of the elementary pathways upon assembly, and lastly, the global level acts as a continuous switch upon local and elementary metabolic activity by translating the “higher meaning” of physiological state or network topology into regulatory action.

In a mathematical context the cybernetic or control variables reflecting all three levels of control, termed the complete cybernetic variables and are defined functionally by

\[
\begin{align*}
    u_j &= \prod_{k} u_j^k, \\
    v_j &= \prod_{q} v_j^q
\end{align*}
\]

are defined as the local regulatory input, where \(u_j^k\), \(v_j^q\) denote the local cybernetic variables. This postulated level of regulatory input governs the interactions of the elementary pathways stemming from assembly. The functionality of \(u_j^k\) and \(v_j^q\), i.e., the elementary cybernetic variables reflecting the \(k\)th control signal influencing the expression and activity of the \(j\)th enzyme, follows from the solution of the elementary cybernetic resource allocation problem(s) associated with key enzyme \(e_j\). Note that the local component consists of the product of the elementary components because of the possibility that \(e_j\) may be written as a member of multiple elementary pathways, i.e., competes for resources from the resource pool associated with each elementary pathway (overlapping assembly.) The product(s)

\[
\begin{align*}
    u_j^k &= \prod_{q} U_{j,q}, \\
    v_j^q &= \prod_{q} V_{j,q}
\end{align*}
\]

are defined as the global regulatory input influencing the expression and activity of \(e_j\). The functional forms of \(U_{j,q}\), \(V_{j,q}\), (the \(q\)th global signal influencing the expression and activity of the \(j\)th enzyme) follow from the specifics of the global regulatory communication.

**Definition 3.2.** The elementary cybernetic allocation problem is the optimal resource allocation problem associated with the elements in the pathway “library.” In particular, each member of the pathway “library” possess an objective function which is subject to constraint on the level of resources that are allocated for pathway operation. Let us denote the objective and resource constraint associated with the \(k\)th elementary pathway by

\[
\max \{ f_k(x_k) \} \text{ subject to } g_k = \sum_{x} x^k = X_k, \forall j.
\]
where \( x_k^z \in X_k \equiv \{ x_1^z, x_2^z, ..., x_n^z \} \) denotes the resource allocated to the \( z \)th alternative competing in the \( k \)th elementary pathway. The functional form of the elementary cybernetic variable \( u_k^j \), i.e., the control variable that governs the allocation of critical resources from the \( k \)th elementary resource pool for the expression of \( e_j \) follows from the matching law optimization of (3.34). The functional form of the elementary cybernetic variable that describes the regulation of the activity of \( e_j \) stemming from the \( k \)th elementary pathway follows from the cybernetic proportional law.

**Lemma 3.1.** The objective of a metabolic network follows as a consequence of the structure of the local regulatory aspect. In particular, \( u_k^j \) stems from the solution of the cybernetic resource allocation problem associated with the \( k \)th elementary pathway in isolation. This implies that the local regulatory element represents the “fusion” of elementary objectives and moreover that the objective associated \( e_j \) expression is the disjoint objective set

\[
\max \{ f_j(x_k) \} \text{ and } \max \{ f_{j+1}(x_{k+1}) \} \text{ and } \ldots \text{ and } \\
\max \{ f_k(x_k) \} \text{ and } \ldots \text{ and } \max \{ f_{k+q}(x_{k+q}) \} 
\]

subject to the disjoint constraint set

\[
g_j(x_k) \equiv X_s \text{ and } g_{j+1}(x_{k+1}) \equiv X_{s+1} \text{ and } \ldots \text{ and } \\
g_k(x_k) \equiv X_s \text{ and } \ldots \text{ and } g_{k+q}(x_{k+q}) \equiv X_{s+q},
\]

where \( e_j \) is a member of \( q \) elementary pathways. The influence of global control then acts as an additional constraint upon local allocation policy by modifying the local regulatory element in the complete cybernetic variable.

3.1.2. Extension of the Cybernetic Framework to Recombinant Systems

Now that we have reviewed the basis of the modular approach, the final item that must be addressed before formulating and evaluating the aspartate model system is the extension of cybernetic principles to recombinant systems. At first glance these concepts may seem incompatible, however, in fact the introduction of genetic changes to metabolic networks and moreover the subsequent “reaction” of the network is intimately integrated with the hypothesis that metabolic pathways are goal oriented. We present a series of postulates that form the basis for the extension. In particular, we begin by presenting the basic concepts that underlie the optimality of the cybernetic framework. We then reexamine these postulates in light of overexpression and deletion of existing pathway enzymes.

The core hypothesis of the cybernetic framework is that, because of evolutionary pressure, the microorganism has evolved control machinery that steers the nutritional state toward an objective in an optimal manner. The control action takes the form of the regulation of expression and activity of the enzymes that constitute the network. Previous cybernetic investigators have assumed, when formulating abstracted models of microbial processes, the maximization of growth rate as an evolutionary objective. However, in the context of metabolic engineering, although such an objective may be operating at a suitably global level, the local objective of a metabolic network is postulated to be a non-unique function of the pathway topology, i.e., the network objective follows from the interaction of the individual elementary pathway objectives.

These two schools of thought are not inconsistent, even when they appear to be contradictory, for example in the case of storage metabolism. Often the product of metabolic function is required for growth, i.e., specific intermediates, energetic metabolites, etc. Thus, a postulated objective of a metabolic network is local in the sense that it is a suitable refinement of the macroscopic growth rate postulate. In the case of a seemingly contradictory objectives, it must be remembered that global objectives may be nutritional state dependent (as is true of storage metabolism or maintenance function.) In these instances the objective of nongrowth related metabolic networks is still a function of the topology, however, the nutritional state in some measure influences the network startup, i.e., the network is subject to global nutritional control. Whichever the case may be, the metabolic control system that regulates the network, evaluates the existing nutritional alternatives and directs the synthesis of the network enzymes for the set of alternatives that yield the optimum nutritional outcome. We postulate that for a genetically modified network, this process is altered slightly, however, in spirit remains unchanged.

**Postulate 3.1.** The objective of a base network is still in place for a genetically modified version of the same network.

This postulate implies that overexpression or deletion of existing pathway enzymes does nothing to change the objective of the network. In other words, the base network objective is in place because of the force of millions of years of evolutionary pressure, and as such, can not simply be immediately erased by a small number of perturbations. Overexpression or deletion of enzymatic machinery does of course, influence the performance of the pathway, and moreover, the choice operational functionality, i.e., which particular enzymes are expressed and the degree of promotion in each case because now the microorganism faces a modified set of operational alternatives.

**Postulate 3.2.** The metabolic control system, in the case of the modified network, must cope with an expanded or...
contracted set of possible alternatives reflecting overexpression or deletion of network enzymes when regulating the synthesis and activity of existing network enzymes.

In short, it is hypothesized that the objective function of the base network is still in place even in the case of the recombinant system. However, the available set of alternatives that are open to the organism for the case of the genetically modified network is different. Thus, from a resource allocation perspective, the result of genetic modifications to the network (deletion or overexpression) is the contraction or expansion of the set of alternatives that are available to the microorganism, not the alteration of the objective itself. Accordingly, the allocation of critical resources for network operation is still taken to be optimal, however, the set of alternatives that compete for these resources is modified. In simpler terms, these arguments imply that base network control machinery directs metabolic function towards the base objective despite the genetic alteration. Clearly, however, the base case and the altered case are not the same. More precisely, the intact base regulatory machinery still directs function toward the base network objective in an optimal manner, however, it does so in light of the genetic perturbation. In a formal sense, we maintain

**Postulate 3.3.** Functionality shifts observed in response to genetic perturbation are an attempt on the part of the network control machinery to maintain the base objective as best possible in the face of changes forced upon the system by genetic alteration.

In a cybernetic sense, the deletion of existing pathway enzymes reduces the number of alternatives that compete for key cellular resources. Thus, inclusion of this class of genetic manipulation into the current framework is straightforward.

**Postulate 3.4.** Let $u^k_j$ denote the elementary cybernetic variable that governs the allocation of critical resources form the $k$th pool to the $j$th network enzyme. Now suppose the gene that encodes for $e_j$ has been deleted. The $j$th enzyme no longer competes for key cellular resource from the $k$th resource pool or any other which implies

$$u^k_j = 0.$$  \hfill (3.37)

**Remark 3.1.** An entirely equivalent description of the ramifications of gene deletion follows by assuming the rate constant governing the expression of enzyme $e_j$ is defined as

$$x_{e_j} = \begin{cases} \tilde{x}_{e_j} & \text{gene encoding for } e_j \text{ present} \\ 0 & \text{gene encoding for } e_j \text{ deleted.} \end{cases}$$  \hfill (3.38)

This representation implies $e_j = 0 \forall t \Rightarrow r_j = 0$ if the gene that encodes for $e_j$ has been deleted, thus, the expression of $e_j$ does not compete for cellular resources because allocation is based upon the rate of return, as measured by reaction rate.

Another possible route of genetic manipulation is the mutation of a gene which encodes for an enzyme such that a normal expression profile is observed, however, the enzyme is capable of only a fraction of its native activity.

**Postulate 3.5.** Let $v^k_j$ denote the elementary cybernetic variable that regulates the activity of the $j$th enzyme of the $k$th elementary pathway. Suppose the gene which encodes for $e_j$ is altered such that $e_j$ is only capable of a fraction of its wild type activity. The modified elementary cybernetic variable that reflects this contingency, denoted by $v^k_j$, is of the form

$$v^k_j = \phi^k_j v^k_j, \quad 0 \leq \phi^k_j \leq 1,$$  \hfill (3.39)

where $\phi^k_j$ denotes the fraction of maximum of activity that the modified $e_j$ is capable of. A value of $\phi^k_j = 1$ denotes the wild type activity, whereas, $\phi^k_j = 0$ indicates a completely blocked enzymatic step.

**Remark 3.2.** Note that postulate (3.5) assumes a normal expression profile, i.e., the expression of the $j$th gene that encodes for $e_j$ is identical to that of the wild-type network. This implies that the alteration that leads to decreased activity is not in the promoter region. If this is not true, i.e., the expression profile and the activity of $e_j$ are altered, then in addition to postulate (3.5) the rate and saturation constant(s) that govern $e_j$ expression can be altered to reflect the new expression profile.

**Remark 3.3.** Given the assumption of a normal expression profile, the completely blocked case ($\phi^k_j = 0$) is not equivalent to postulate (3.4) or remark (3.1) because we assume $e_j$ is still expressed but has no activity.

The deregulation of existing network enzymes can also be described within the framework, i.e., the removal of metabolite feedback inhibition/repression. Note, within the present context, these signals are classified as global nutritional signals, thus, we frame the discussion around the more general topic of removal of global regulatory sensitivity.

**Postulate 3.6.** Suppose the enzyme $e_j$ is a member of the $k$th elementary pathway and is subject to $q$ global control signals. The complete cybernetic variable that regulates the expression of $e_j$ is given by

$$u_j = u^j(U_1, U_2, \ldots, U_q),$$  \hfill (3.40)
where \( u_j \) denotes the local regulatory component. Now suppose, as a consequence of an unspecified mutation, \( e_j \) is no longer sensitive to the \( r \)th global control signal. This alteration is reflected by assuming

\[
U_r \equiv 1. \tag{3.41}
\]

This implies the modified complete cybernetic variable that regulates that expression of \( e_j \) is given by

\[
u_j = u_j(U_1 U_2 \ldots U_q). \tag{3.42}
\]

**Remark 3.4.** The postulate above describes the removal of a global sensitivity connected with enzyme expression. The removal of the global control of enzyme activity follows by analogy.

The representation of the overexpression of existing pathway enzymes is slightly more involved because of the possibility of multiple expression sources.

**Postulate 3.7.** The overexpression of existing pathway enzymes is a substitutable process. Enzyme expression stemming from the genome and plasmid form a substitutable pair in the cybernetic sense, i.e., both routes compete for key cellular resource to synthesize a common product.

Postulate (3.7) implies the existence of a cybernetic control variable that regulates the allocation of key resources between possible expression sources. Because the native and plasmid expression routes are unaware of the existence of one another the control action that regulates the choice of synthesis route is classified as a global signal.

**Postulate 3.8.** There exists a global cybernetic variable, denoted as \( U^* \), \( k = g, p \), that regulates the allocation of critical resources to each individual expression route. The global control variable \( U^* \), \( k = g, p \) where superscript(s) \( g, p \) denote genome and plasmid, respectively, reflects the substitutable nature of overexpression.

To derive the form of these control variables we pose a problem similar to the elementary convergent pathway. Specifically, there exist two routes for the synthesis of the common product, namely, enzyme \( e_j \). Both routes require critical cellular resources to proceed. Accordingly, we postulate that the local objective of enzymatic expression (barring any global interaction, such as nutritional state dependent synthesis) is the maximization of the level of key enzyme \( e_j \), subject to a constraint upon the amount resources available for synthesis of \( e_j \). These statements formally become the constrained optimization problem

\[
\max \left\{ \sum_j c_j(R_i) \right\} \quad \text{subject to} \quad g = \sum_j R_i = R_i. \tag{3.43}
\]

where \( R_i \) denotes the resource allocated to the \( r \)th route of synthesis of enzyme \( e_j \), \( n \) denotes the total number of possible routes, and \( c_j \) denotes the level of enzyme \( e_j \) synthesized via the \( r \)th route. The optimality condition of the constrained maximization problem given by

\[
\frac{dc_j}{dR_1} = \frac{dc_j}{dR_2} = \cdots = \frac{dc_j}{dR_{n-1}} = \frac{dc_j}{dR_n} \tag{3.44}
\]

can be rearranged to yield the matching criteria

\[
\frac{dc_j}{\sum_{k=1}^{n-1} dc_j} = \frac{dR_i}{\sum_{k=1}^{n-1} dR_k}, \quad \forall i. \tag{3.45}
\]

Equation (3.45) is a restatement of the matching law criteria put forth by other cybernetic investigators. In short, it stipulates that the optimum allocation policy is one in which the fractional allocation of critical resources is equal to the fractional return on investment. If the allocation policy is implemented at every instant in time and takes place in time \( dt \), the return on investment can be measured as the rate of enzyme synthesis. In the case in which the synthesis route is chromosomal, the rate of enzyme expression is given by the regulated rate, i.e.,

\[
r_{ej} u_j, \quad \forall j \tag{3.46}
\]

whereas enzyme expression via the plasmid is measured as

\[
r_p e, \quad \forall e \tag{3.47}
\]

whose form is dependent upon the promoter type. The inclusion of the regulated rate of chromosomal synthesis follows form the postulate that the enzyme synthesis routes are completely functional and independent. Within the present context, we are considering the competition between the chromosomal synthesis route and expression via a plasmid of the key enzyme \( e_j \). Accordingly, it follows from Eq. (3.45) that the cybernetic variables which govern the allocation of critical resources for the synthesis of key enzyme \( e_j \) from the genome and plasmid are given functionally as

\[
U_e = \frac{r_{ej} u_j}{r_{ej} u_j + r_e^p} \quad U^p_e = \frac{r_e^p}{r_{ej} u_j + r_e^p}. \tag{3.48}
\]

where the superscripts \( e, p \) denote genome and plasmid, respectively. The over-expression of \( e_j \) is accounted for within the model equations by replacing the term \( r_{ej} u_j \) in the \( e_j \) balance with

\[
r_{ej} u_j U_e + r_e^p U^p_e. \tag{3.49}
\]
where the second synthesis rate stems from expression via the plasmid. Note the presence of the control variables $U_{ej}^k$, $k = g, p$. These act as a global control mechanism upon enzyme synthesis that regulates the source of synthesis of $e_j$.

In simpler terms, $U_{ej}^k$ is equivalent to the fraction of cellular resources allocated to $e_j$ expression being directed to the $k$th synthesis source. For example, suppose the native route (in the absence of synthesis of $e_j$ from the plasmid) is highly favored, i.e., $u_j = 1$. Furthermore, suppose the synthesis rate from the plasmid is much greater than chromosomal synthesis rate and under the control of an artificial promoter. Before addition of inducing agent, $r_{ej}^p = 0$ which implies $U_{ej}^p = 0$ and $U_{ej}^g = 1$, i.e., the native expression route is the only active synthesis route for $e_j$ therefore it consumes all resources allocated to $e_j$ expression. After addition of the inducing agent, because of the assumption $r_{ej}^p > r_{ej}^g$, the majority of enzyme synthesis is from the plasmid, i.e., the plasmid route is faster than synthesis from the genome, accordingly, it is allocated a larger portion of the critical resource pools. The overexpression of key enzymes is modulated, the ramifications ripple

3.2. Derivation: Elementary Cybernetic Variables

As per the discussion above, the cybernetic regulation will consist of three levels, namely, an elementary, local and a global regulatory component. The local component is derived from the topological nature of the pathway, i.e., follows from the set of elementary building blocks used to realize the pathway topology, whereas, the global component describes the higher regulatory intentions of the organism, in this instance, metabolite feedback sensitivity. We model the aspartic-$\beta$-semialdehyde and homoserine branch points as flexible as discussed by Stephanopoulos and Vallino (Stephanopoulos and Vallino, 1991). This implies that no direct regulatory interaction exists between the branch metabolites, rather, they interact only through kinetic competition for the common branch precursor metabolite. It follows from the previous development regarding the cybernetic representation of metabolic branch points (Varner and Ramkrishna (Varner and Ramkrishna, 1998b)) that the local regulatory component consists of three overlapping elementary linear pathways, with end products $p_5^a$, $p_8^b$ and $p_8$, respectively. The metabolic network objective, given this regulatory formulation, is the maximization of the elementary linear pathway end products, namely, $p_5^a$, $p_8^b$ and $p_8$ subject to the level of resource allocated for function of the corresponding elementary pathway. Note because the elementary pathway overlap, the shared elements between elementary pathways compete for resources from multiple pools. The interesting biological implication of such an observation is that the synthesis and the activity of the key enzymes that contribute to the formation of the network intermediates are coupled via this resource sharing. Thus, as resource competitiveness of key enzymes is modulated, the ramifications ripple
throughout the network because of competition for key cellular resources. The global regulatory component which describes metabolite repression/inhibition then acts to constrain the local allocation policy or local enzyme activity, respectively.

The network under consideration is composed of three overlapping elementary linear pathways. The primary elementary pathway, termed the backbone pathway, extends from $p_0$ to $p_8$ and is denoted as elementary pathway $c$. The two remaining elementary pathways, i.e., from $p_6$ to $p_8$ and $p_0$ to $p_4$ are secondary elementary branch pathways and are denoted as $a, b$, respectively. Note that each of these pathways is linear, thus, the elementary cybernetic problem associated with each is identical.

The objective function of the elementary linear pathway is postulated, consistent with Straight and Ramkrishna (Straight and Ramkrishna, 1994), to be the maximization of the end product subject to a constraint upon the level of resources allocated for elementary pathway function. Accordingly, the constrained optimization problem associated with the $k$th $n(k)$-dimensional linear pathway

$$
\max \{ p^k_n(R^k_n-1, p^k_{n-1}(R^k_{n-2}, \ldots, p^k_1(R^k_n))) \}
$$

subject to

$$
g_k = \sum_{j=0}^{n} R^k_j = R_k, \ \forall k
$$

possesses the optimality condition

$$
dp^k_n = \frac{dp^k_{n-1}}{dR^k_{n-1}} - \frac{dp^k_{n-2}}{dR^k_{n-2}} = \ldots = \frac{dp^k_1}{dR^k_0} \forall k,
$$

where

$$
a_{j, j-1, k} \equiv \frac{dp^k_j}{dp^k_{j-1}}, \ \forall k, j
$$

denotes the stoichiometric coefficient(s) relating the conversion

$$
p_{j-1} \xrightarrow{a_{j, j-1, k}} p_j
$$

in the $k$th elementary linear pathway. Optimality condition (3.51) can be rearranged to yield the set of matching criteria

$$
\frac{dp^k_n}{dR^k_n} = \frac{dp^k_{n-1}}{dR^k_{n-1}} + \frac{dp^k_{n-2}}{dR^k_{n-2}} + \ldots + \frac{dp^k_1}{dR^k_0}
$$

subject to $\frac{dR^k_n}{\sum_{j=0}^{n-2} dR^k_j}$

$$
a_{j, j-1, k} \equiv \frac{dp^k_j}{dp^k_{j-1}}, \ \forall k, j
$$

where

$$
a_{j, j-1, k} \equiv \frac{dp^k_j}{dp^k_{j-1}}, \ \forall k, j
$$

The matching condition(s) shown above simply state that optimum pathway operation with respect to resource allocation comes about when the fractional return on investment is equal to the fractional allocation of pathway resources. If we assume that the optimum allocation policy is implemented at every instant of time and that allocation takes place on the time scale of $dt$, then the return on investment can be measured as the reaction rate. Accordingly, the cybernetic variable that governs the allocation of critical resources from the $k$th elementary resource pool to the synthesis of $j$th key enzyme belonging the $k$th elementary linear pathway, denoted as $u^k_j$, is given by

$$
u^k_j = \frac{a_{n, j, k} \nu^k_j}{r^k_{n-1} + a_{n, n-1, j}r^k_{n-2} + \ldots + a_{n, j, 1}r^k_j + \ldots + a_{n, 1}r^k_0},
$$

where

$$
0 \leq \nu^k_j \leq 1, \ \forall k, j.
$$

The elementary cybernetic variable that governs the activity of the key enzyme catalyzing the $j$th metabolic transformation of the $k$th elementary pathway, denoted as $v^k_j$ follows from the cybernetic proportional law. In short, this law postulates that enzyme activity is proportional to reaction rate. Moreover, it is postulated that the key enzyme which catalyzes the maximum rate amongst the set of rates competing in the $k$th elementary pathway possess the maximal activity. These postulates imply

$$
v^k_j \sim \lambda^k_j \nu^k_j, \ \forall k, j.
$$

The cybernetic variable $v^k_j$ is constrained by definition to lie in the interval

$$
0 \leq v^k_j \leq 1, \ \forall k, j
$$
The proportionality constant \( \lambda_k \) must obey equation (3.59) for all \( j \) in the \( k \)th elementary pathway, thus, \( \lambda_k \) takes the form
\[
\lambda_k = \frac{1}{\max(r^k_1, r^k_2, \ldots, r^k_n)}
\]
which implies \( e^j_k \) is of the form
\[
e^j_k = \frac{r^k_j}{\max(r^k_1, r^k_2, \ldots, r^k_n)}, \quad \forall k, j.
\]

3.3. Derivation: Complete Cybernetic Variable

The key enzyme \( e_0 \) is a member of all three elementary linear pathways which implies that it can receive resources from three different independent resource pools. Additionally, the key enzyme \( e_0 \) is subject to concerted feedback inhibition by the branch metabolite \( p^*_3 \) and the intermediate \( p_5 \). The elementary cybernetic variable governing the allocation of critical resources for the synthesis of \( e_0 \) from the \( j \)th pool is given functionally as
\[
u^j_0 = \frac{r_0}{\sum_{k(j)} r_k}, \quad j = a, b, c.
\]
where
\[
\sum_{k(a)} r_k = r_0 + r_1 + r^*_2,
\]
\[
\sum_{k(b)} r_k = r_0 + r_1 + r^*_2 + r^*_3,
\]
\[
\sum_{k(c)} r_k = r_0 + r_1 + r^*_2 + r^*_3 + \sum_{q=4}^8 r_q.
\]
The elementary cybernetic variable which governs the activity of \( e_0 \) (note that \( e_0 \) is a member of three elementary pathways) is given by the set
\[
u^j_0 = \frac{r_0}{\max(r^j_k)}, \quad j = a, b, c,
\]
where the local competition set \( \{r^j_k\} \) is given by
\[
\{r^j_k\} = \{r_0, r_1, r^*_2\},
\]
\[
\{r^j_k\} = \{r_0, r_1, r^*_2, r^*_3\},
\]
\[
\{r^j_k\} = \{r_0, r_1, r^*_2, r^*_3, \{r^*_q\}_{q=4}^8\}.
\]
The local cybernetic variable that governs the expression of \( e_0 \) is the product of the elementary variables. This follows from the possibility of allocation from multiple resource pools. The local cybernetic variable which governs \( e_0 \) synthesis is given by
\[
u^j_0 = u^j_0 u^j_0 u_0^j.
\]
Note because expression of \( e_0 \) is not sensitive to any global regulatory signals \( u^j_0 \) describes all regulatory input to the synthesis of \( e_0 \). In other words, the complete cybernetic variable that controls the expression of \( e_0 \) is given by
\[
u^j_0 = u^j_0 u^j_0 u_0^j.
\]
The local cybernetic variable which governs enzyme activity follows from above and is given by
\[
u^j_0 = v^j_0 k^j_0 v^j_0.
\]
The key enzyme \( e_0 \) is sensitive to concerted feedback inhibition by the network metabolites \( p^*_3 \) and \( p_5 \). This signal is represented as a global control upon local activity. The derivation of the global control variables follows from the arguments presented in the previous members of this series. Namely, we postulate the inhibition of \( e_0 \) is proportional to the level of the intermediates \( p^*_3 \) and \( p_5 \), i.e.,
\[
V^3_l \sim \lambda \psi^{V^3}_{e_0 p^*_3 p^*_3} V^5_l \sim \lambda \psi^{V^5}_{e_0 p_5 p^*_3} \max
\]
where \( \psi^{V^3}_{e_0 p^*_3 p^*_3} \) and \( \psi^{V^5}_{e_0 p_5 p^*_3} \) denote the minimum level of \( p^*_3, p_5 \) at which \( e_0 \) has zero activity where \( 0 < \psi^{V^3}_{e_0 p^*_3 p^*_3} \leq 1 \), \( 0 < \psi^{V^5}_{e_0 p_5 p^*_3} \leq 1 \) and \( p^*_3 \) and \( p^*_3 \) denote the maximum biotic phase level of \( p^*_3 \) and \( p_5 \), respectively. The terms \( \psi^{V^3}_{e_0 p^*_3 p^*_3}, j = p^*_3, p_5 \) are defined as the minimum fraction of the maximum biotic phase metabolite level at which complete inhibition of \( e_0 \) occurs, i.e., the complete inhibition threshold. Clearly, when \( p^*_3 = p_5 = 0 \) \( e_0 \) experiences no inhibitory effects, i.e., \( V^3_0 \rightarrow 0 \) as \( p^*_3 \), \( p_5 \rightarrow 0 \). Furthermore, \( V^3_0 \) is given when \( p^*_3 \) and \( p_5 \) are greater than or equal to the
complete inhibition threshold. These statements imply the functional form

\[ V_{0}^{5} = \begin{cases} 
1 - \frac{p_{5}^{a}}{\psi_{p_{5}}^{c} p_{5}^{\max}} & p_{5}^{a} < \psi_{p_{5}}^{c} p_{5}^{\max} \\
0 & p_{5}^{a} \geq \psi_{p_{5}}^{c} p_{5}^{\max}
\end{cases} \]  (3.68)

and

\[ V_{0}^{6} = \begin{cases} 
1 - \frac{p_{6}^{a}}{\psi_{p_{6}}^{c} p_{6}^{\max}} & p_{6}^{a} < \psi_{p_{6}}^{c} p_{6}^{\max} \\
0 & p_{6}^{a} \geq \psi_{p_{6}}^{c} p_{6}^{\max}
\end{cases} \]  (3.69)

The complete cybernetic variable governing the activity of \( e_{0} \) is the product of the local and global activity components and is given by

\[ v_{0} = v_{0}^{1} V_{0}^{5} V_{0}^{6}, \]  (3.70)

The key enzyme \( e_{1} \) is a member of all three elementary pathways, however, is not sensitive to any global regulatory input. The local cybernetic variable that governs resource allocation is the product of the elementary pathway allocation variables given by

\[ u_{j} = \frac{r_{j}}{\sum_{k \in (j)} r_{k}}, \quad j = a, b, c, \]  (3.71)

where

\[ \sum_{k \in (a)} r_{k} = r_{0} + r_{1} + r_{3}^{a}, \]
\[ \sum_{k \in (b)} r_{k} = r_{0} + r_{1} + r_{3}^{b} + r_{3}^{ba}, \]
\[ \sum_{k \in (c)} r_{k} = r_{0} + r_{1} + r_{3}^{c} + r_{3}^{bc} + \sum_{q = 4}^{8} r_{q}. \]

It follows that the local cybernetic variable that controls the synthesis of \( e_{1} \) take the form

\[ u_{1}^{1} = u_{1}^{4} u_{1}^{2} u_{1}^{1}. \]  (3.72)

Moreover, because \( e_{1} \) is not sensitive to any global regulatory signal, the complete cybernetic variable that controls the expression of \( e_{1} \) is given by

\[ u_{1} = u_{1}^{1}. \]  (3.73)

The functional form of the cybernetic variable that controls enzyme activity follows along the same lines as shown above. More exactly, because the key enzyme \( e_{1} \) is a member of three elementary pathways the local cybernetic regulatory component is the product of elementary components. The elementary cybernetic variables which govern the activity of \( e_{1} \) are given by the set

\[ e_{j}^{1} = \frac{r_{j}}{\max(r_{k}), j = a, b, c,} \]  (3.74)

where the local competition set \( \{r_{k(j)}\} \) is given by

\[ \{r_{k(a)}\} = \{r_{0}, r_{1}, r_{3}^{a}\}, \]
\[ \{r_{k(b)}\} = \{r_{0}, r_{1}, r_{3}^{b}, r_{3}^{ba}\}, \]
\[ \{r_{k(c)}\} = \{r_{0}, r_{1}, r_{3}^{c}, r_{3}^{bc}, r_{4}, r_{q}, q = 4\}. \]

The local regulatory component that describes enzyme activity is given by

\[ e_{1} = e_{1}^{1} e_{1}^{2} e_{1}^{3}. \]  (3.75)

Because \( e_{1} \) is not subject to any global regulatory signal, the complete cybernetic variable that describes the activity of \( e_{1} \) is given by

\[ v_{1} = v_{1}^{1}. \]  (3.76)

The key branch point enzyme \( e_{2}^{b} \) is a member of only a single elementary pathway and is not subject to any global regulatory signal. Thus, the regulation associated with \( e_{2}^{b} \) is composed of only the elementary regulatory component. The elementary cybernetic variable governing the synthesis of \( e_{2}^{b} \) is of the form

\[ u_{2}^{b} = u_{2}^{b} = \frac{r_{2}^{b}}{r_{0} + r_{1} + r_{2}^{b}}. \]  (3.77)

It follows the complete cybernetic variable that controls the synthesis of \( e_{2}^{b} \) takes the form

\[ u_{2} = u_{2}^{1} = u_{2}^{b}. \]  (3.78)

The activity of \( e_{2}^{b} \) also follows solely from the elementary regulatory component which is given by

\[ e_{2}^{1} = \frac{r_{2}^{b}}{\max(r_{0}, r_{1}, r_{2}^{b})}. \]  (3.79)

Thus, the complete cybernetic variable which describes the activity of \( e_{2}^{b} \) is of the form

\[ v_{2} = v_{2}^{1} = v_{2}^{b}. \]  (3.80)
The key enzyme $e_5^k$ is a member of two elementary pathways and is subject to a inhibitory signal emanating from $p_3^k$ and a repressive signal emanating from $p_4^k$. The local regulatory component that describes the regulation of $e_5^k$ expression is the product of the elementary regulatory influences. The elementary regulatory component governing $e_5^k$ expression is described by the elementary cybernetic variables

$$u_{2b}^j = \frac{r_p^b}{\sum_{k(i)} r_k}, \quad j = b, c, \quad (3.81)$$

where

$$\sum_{k(h)} r_k = r_0 + r_1 + r_2^h + r_3^h,$$

$$\sum_{k(v)} r_k = r_0 + r_1 + r_2 + r_3 + \sum_{q=4}^8 r_q.$$

It follows, because $e_5^k$ can receive resources from two independent elementary pools, that the local cybernetic regulatory component is the product of the elementary components i.e.,

$$u_{2b}^l = u_{2b}^p u_{2b}^c. \quad (3.82)$$

The local component is subject to the repressive influence of the intermediate $p_4^h$. Thus, the allocation policy of critical resource for the synthesis of $e_5^k$ must be modified by a global component to make it sensitive to the repression. The form of the global control variable follows from the discussion above. Let $\psi_{e_5^k, p_4^h, p_4^h, \text{max}}$ denote the minimum level of $p_4^h$ where $e_5^k$ expression is completely repressed. It follows that at this level of $p_4^h$, the global control variable denoted as $U_{2b}^{\text{max}}$ assumes a value of zero. Additionally when $p_4^h = 0$, the key enzyme $e_5^k$ should feel no repressive effect. Accordingly, we postulate the global control variable is proportional to the level of $p_4^h$ i.e.,

$$U_{2b}^{\text{max}} \sim \psi_{e_5^k, p_4^h, p_4^h, \text{max}} (p_4^h - \psi_{e_5^k, p_4^h, p_4^h, \text{max}}) \quad (3.83)$$

and after some simple algebraic manipulation we arrive at the functional form

$$U_{2b}^{\text{max}} = \begin{cases} 1 - \frac{p_4^h}{\psi_{e_5^k, p_4^h, \text{max}}} & p_4^h < \psi_{e_5^k, p_4^h, \text{max}} \\ 0 & p_4^h \geq \psi_{e_5^k, p_4^h, \text{max}} \end{cases} \quad (3.84)$$

The complete cybernetic variable that describes the control of the synthesis of $e_5^k$ is the product of the local and global regulatory components given by

$$u_{2b} = u_{2b}^l U_{2b}^{\text{max}}. \quad (3.85)$$

The control of enzyme activity follows from the discussion above. Because $e_5^k$ is a member of two elementary pathways the local regulatory component is the product of the elementary cybernetic variables given by

$$v_{2b}^j = \frac{r_p^b}{\max\{r_{ki(j)}\}}, \quad j = b, c, \quad (3.86)$$

where the competition set $\{r_{ki(j)}\}$ is given by

$$\{r_{ki(h)}\} = \{r_0, r_1, r_2^h, r_3^h\},$$

$$\{r_{ki(v)}\} = \{r_0, r_1, r_2, r_3, \{r_q\}_{q=4}^8\}.$$

It follows that the local regulatory component is given by

$$v_{2b}^l = v_{2b}^p v_{2b}^c. \quad (3.87)$$

The activity of the key enzyme $e_5^k$ is sensitive to the levels of the pathway intermediate $p_3$. Thus, the local cybernetic component governing enzyme activity must be modified by a global control variable so as to account for this sensitivity. The functional form of the global control variable follows from arguments presented earlier and is given by

$$V_{2b}^m = \begin{cases} 1 - \frac{p_4^h}{\psi_{e_5^k, p_4^h, p_4^h, \text{max}}} & p_4^h < \psi_{e_5^k, p_4^h, \text{max}} \\ 0 & p_4^h \geq \psi_{e_5^k, p_4^h, \text{max}} \end{cases} \quad (3.88)$$

The complete cybernetic variable governing the activity of key enzyme $e_5^k$ is the product of the local and global regulatory components and takes the form

$$v_{2b} = v_{2b}^l V_{2b}^m. \quad (3.89)$$

The key enzyme $e_5^h$ is a member of only a single elementary pathway and is sensitive to the level of $p_4^h$. The elementary regulatory component governing the allocation of critical resources for the synthesis of $e_5^h$ is given by

$$u_{3bh} = u_{3bh}^l = \frac{r_p^h}{r_0 + r_1 + r_2 + r_3}. \quad (3.90)$$

Note because $e_5^h$ is a member of only a single elementary pathway the local and elementary components are identical.
The complete cybernetic variable governing the synthesis of \( e_s^{bb} \) is given by

$$ u_{sba} = u_{sba}^i = u_{sba}^b. \tag{3.91} $$

The activity of \( e_s^{bb} \) is subject to both local as well as global control. It follows because \( e_s^{bb} \) is a member of only a single linear pathway that the local and elementary regulatory components are identical i.e.,

$$ v_{sba}^l = v_{sba}^i = \frac{r_{sba}^b}{\max(r_0, r_1, r_2, r_3, r_{sba}^b)}. \tag{3.92} $$

The activity of the key enzyme \( e_s^{bb} \) is sensitive to the level of the intermediate \( p_{s5}^{ba} \). The form of the global control variable which describes this sensitivity follows from the previous discussion and is given functionally by

$$ v_{sba}^g = \left\{ \begin{array}{ll}
1 - \frac{p_{s5}^{ba}}{\psi_{s5}^{ba}, p_{s5}^{ba, \max}} & p_{s5}^{ba} < \psi_{s5}^{ba}, p_{s5}^{ba, \max} \\
0 & p_{s5}^{ba} \geq \psi_{s5}^{ba}, p_{s5}^{ba, \max}.
\end{array} \right. \tag{3.93} $$

The complete regulatory component is the product of the local and global regulatory elements and is given by

$$ v_{sba} = v_{sba}^l V_{sba}^{sba}. \tag{3.94} $$

The key enzyme \( e_s^{bb} \) is a member of a single elementary pathway and is sensitive to levels of \( p_{s5} \). The elementary cybernetic variable which governs the synthesis of \( e_s^{bb} \) is given by

$$ u_{sba}^{bb} = \frac{r_{sba}^{bb}}{r_0 + r_1 + r_2 + r_3 + r_{sba}^{bb} + \sum_{k=4}^8 f_k}. \tag{3.95} $$

Because \( e_s^{bb} \) is a member of only a single elementary pathway, the local cybernetic regulatory component that governs \( e_s^{bb} \) synthesis is given by

$$ u_{sba}^l = u_{sba}^{bb}. \tag{3.96} $$

Moreover, because \( e_s^{bb} \) expression is not sensitive to global signals, the complete cybernetic variable governing the synthesis of the key enzyme \( e_s^{bb} \) is given by the local regulatory component

$$ u_{sbb} = u_{sbb}^l. \tag{3.97} $$

The elementary cybernetic variable governing the activity of \( e_s^{bb} \) is given functionally as

$$ v_{sbb}^l = v_{sbb}^i = \frac{r_{sba}^{bb}}{\max(\{r_{bb,c}\})}. \tag{3.98} $$

where the competition set \( \{r_{bb,c}\} \) is given by

$$ \{r_{bb,c}\} = \{r_0, r_1, r_2, r_3, r_4\}^{q=4}. \tag{3.99} $$

Because \( e_s^{bb} \) is a member of only a single linear pathway the local regulatory element that governs \( e_s^{bb} \) activity is the elementary component i.e.,

$$ v_{sbb}^l = v_{sbb}^i. \tag{3.100} $$

The activity of \( e_s^{bb} \) is also sensitive to the level of \( p_{s5} \). Following the previous development, the global control variable that describes the inhibition of \( e_s^{bb} \) with respect to \( p_{s5}^{bb} \) is given functionally as

$$ v_{p_{s5}}^{bb} = \left\{ \begin{array}{ll}
1 - \frac{p_{s5}}{\psi_{s5}^{bb}, p_{s5}^{bb, \max}} & p_{s5} < \psi_{s5}^{bb}, p_{s5}^{bb, \max} \\
0 & p_{s5} \geq \psi_{s5}^{bb}, p_{s5}^{bb, \max}.
\end{array} \right. \tag{3.101} $$

The complete cybernetic variable which governs the activity of \( e_s^{bb} \) consists of the product of the local and global regulatory components and is given by

$$ v_{sbb} = v_{sbb}^l V_{sbb}^{p_{s5}}. \tag{3.102} $$

The key enzyme \( e_s \) is a member of only one linear pathway and is not subject to any global regulatory signals. The elementary component that governs \( e_s \) expression is given by the cybernetic variable

$$ u_{s}^l = \frac{r_{s}}{r_0 + r_1 + r_2 + r_3 + \sum_{k=4}^8 f_k}. \tag{3.103} $$

Because \( e_s \) is member of only a single linear pathway the local regulatory element consists solely of the elementary component. In other words, the local cybernetic regulatory element is given by

$$ u_{sbb} = u_s = u_s^l = u_s^i. \tag{3.104} $$
The elementary cybernetic variable that governs enzyme activity is given by

$$v'_e = \frac{r_e}{\max \{r_{A(e)}\}}. \quad (3.105)$$

where the competition set \( \{r_{A(e)}\} \) is given by

$$\{r_{A(e)}\} = \{r_0, r_1, r_2, r_3, r_4\}. \quad (3.106)$$

Because \( e_5 \) is a member of only a single linear pathway the local regulatory element is composed solely of the single elementary component, i.e.,

$$v'_e = v'_e. \quad (3.107)$$

Moreover, because \( e_5 \) is not subject to any global regulatory signals the complete cybernetic variable that governs the activity of \( e_5 \) is given by

$$v_{468} = v_4 = v'_e. \quad (3.108)$$

The key enzyme \( e_5 \) is a member of only a single elementary pathway and is sensitive to levels of the intermediate \( p_8 \).

The elementary regulatory component that governs the expression of \( e_5 \) is given by

$$u_{5s}' = \frac{r_5}{r_0 + r_1 + r_2 + r_3 + \sum_{k=4}^{8} r_k}. \quad (3.109)$$

The local regulatory element governing the expression of \( e_5 \) is composed solely of the elementary component, i.e.,

$$u'_5 = u'_5. \quad (3.110)$$

Because \( e_5 \) is not sensitive to global regulatory signals, the complete cybernetic variable governing the expression of \( e_5 \) is given by

$$u_5 = u_5. \quad (3.111)$$

The elementary cybernetic variable that controls the activity of \( e_5 \) is given by

$$v'_5 = \frac{r_5}{\max \{r_{A(e)}\}}. \quad (3.112)$$

where the competition set \( \{r_{A(e)}\} \) is given as

$$\{r_{A(e)}\} = \{r_0, r_1, r_2, r_3, r_4\}. \quad (3.113)$$

The local regulatory component consists of solely the elementary cybernetic variable and is given by

$$v'_5 = v'_5. \quad (3.114)$$

The activity of the key enzyme \( e_5 \) is sensitive to levels of the metabolite \( p_8 \). Accordingly, following the previous discussion, the global control variable that describes the inhibition of \( e_5 \) by the metabolite \( p_8 \) takes the form

$$V_{p_8} = \begin{cases} 1 - \frac{p_8}{\psi_p V_{p_8} p_8^{max}} & p_8 < \psi_p U_{e_5} V_{p_8} \, p_8^{max} \\ 0 & p_8 \geq \psi_p U_{e_5} V_{p_8} \, p_8^{max}. \end{cases} \quad (3.115)$$

The complete cybernetic variable which controls the activity of the key enzyme \( e_5 \) is the product of the local and global regulatory components. Therefore, the complete cybernetic variable which controls the activity of \( e_5 \) is given by

$$v_5 = v'_5 \, V_{p_8}. \quad (3.116)$$

The key enzyme \( e_5 \) is a member of only a single linear elementary pathway and is sensitive (both inhibition and repression) to levels of the intermediate \( p_8 \).

The local cybernetic regulatory component which controls the expression of \( e_5 \) is given by

$$u_{5e}' = \frac{r_5}{r_0 + r_1 + r_2 + r_3 + \sum_{k=4}^{8} r_k}. \quad (3.117)$$

Because \( e_5 \) is a member of only a single linear pathway the local regulatory component consists solely of the elementary component, i.e.,

$$u'_5 = u'_5. \quad (3.118)$$

The expression of \( e_5 \) is sensitive to levels of the intermediate \( p_8 \). Following the previous discussion, the global cybernetic variable that describes the repression of \( e_5 \) synthesis by \( p_8 \) takes the form

$$U_{p_8} = \begin{cases} 1 - \frac{p_8}{\psi_p U_{e_5} \, p_8^{max}} & p_8 < \psi_p U_{e_5} V_{p_8} \, p_8^{max} \\ 0 & p_8 \geq \psi_p U_{e_5} V_{p_8} \, p_8^{max}. \end{cases} \quad (3.119)$$

The complete cybernetic variable that controls the expression of \( e_5 \) is the product of the local and global regulatory components and is given functionally as

$$u_5 = u'_5 \, U_{p_8}. \quad (3.120)$$
The elementary cybernetic variables that control the activity of $e_6$ is given functionally as

$$v_6' = \frac{r_6}{\max(\{r_{k(i)}\})},$$

(3.121)

where the competition set $\{r_{k(i)}\}$ is given by

$$\{r_{k(i)}\} = \{r_0, r_1, r_2, r_3, r_4\}.$$  

(3.122)

Because $e_6$ is a member of only a single elementary pathway, the local regulatory component is composed solely of the elementary regulatory element, i.e.,

$$v_6' = v_6^l.$$  

(3.123)

The activity of $e_6$ is sensitive to levels of the pathway intermediate $p_k$. Following the previous discussion, the global control variable which describes the inhibition of $e_6$ by $p_k$ takes the form

$$V_{k}^p = \begin{cases} 1 - \frac{p_k}{\psi_{e_k}, p_{k} P_{k}^{\max}} & p_k < \psi_{e_k}, p_{k} P_{k}^{\max} \\ 0 & p_k \geq \psi_{e_k}, p_{k} P_{k}^{\max} \end{cases}.$$  

(3.124)

The complete cybernetic variable which controls the activity of $e_6$ consists of the product of the local and global regulatory elements and is given functionally as

$$v_6 = v_6^l V_{k}^p.$$  

(3.125)

The key enzyme $e_7$ is a member of a single linear pathway and is not subject to any global regulatory signal. The elementary cybernetic variable which controls the expression of $e_7$ is given by

$$u_7' = \frac{r_7}{r_0 + r_1 + r_2 + r_3 + r_4}.$$  

(3.126)

Because $e_7$ is a member of only a single linear pathway, the local regulatory element consists solely of the elementary component, i.e.,

$$u_7' = u_7^l.$$  

(3.127)

Moreover, $e_7$ synthesis is not subject to a global regulatory signal which implies the complete cybernetic variable that controls the expression of $e_7$ is composed of the local control element, i.e.,

$$u_7 = u_7^l.$$  

(3.128)

The elementary cybernetic variable which controls the activity of $e_7$ is given by

$$v_7' = \frac{r_7}{\max(\{r_{k(i)}\})},$$

(3.129)

where the competition set $\{r_{k(i)}\}$ is given by

$$\{r_{k(i)}\} = \{r_0, r_1, r_2, r_3, r_4\}.$$  

(3.130)

Because $e_7$ is a member of only a single elementary pathway, the local regulatory component is identical to the elementary component, i.e.,

$$v_7' = v_7^l.$$  

(3.131)

Furthermore, because $e_7$ activity is not subject to a global regulatory signal, the complete cybernetic variable which controls the activity of $e_7$ is given by

$$v_7 = v_7'.$$  

(3.132)

### 3.4. Metabolic Regulation: Growth Model

The lumped growth model proposed above is also a cybernetic model thus, we must derive cybernetic variables that regulate the synthesis and activity of the lumped transport and growth enzymes $e_{T_g}$ and $e_g$, respectively. Following the development of Straight and Ramkrishna (Straight and Ramkrishna, 1994), we postulate the objective of the linear growth pathway is the maximization of biomass subject to a constraint on the level of structural resource available for the pathway operation. Formally, this implies the constrained optimization problem

$$\max\{c(R_g, p_g(R_{T,g}))\} \text{ subject to } g = R_g + R_{T,g} = R,$$

(3.133)

where yields the optimality condition

$$\frac{dc}{dR_g} = \frac{dc}{dp_g} \frac{dp_g}{dR_{T,g}}.$$  

(3.134)

The coefficient $dc/dp_g$ denotes the yield of biomass per unit of $p_g$ consumed and is considered constant. If we assume that the allocation of critical structural resource takes place in time $dt$ and the optimal allocation policy is implemented at every instant of time, the return on investment of structural resource can be measured as the reaction rate. Accordingly, the cybernetic variable that controls the
allocation of critical resources to /th step of the growth process, denoted as $u_j$, is given as

$$ u_{T,g} = \frac{Y_{c/g} r_{T,g}}{Y_{c/g} r_{T,g} + r_g} $$

where the yield coefficient $Y_{c/g}$ is equal to $dc/dp_g$. The functional forms of the cybernetic $v$-variables that regulate the activity of the key enzymes that catalyze the growth pathway reactions follow from the proportional law and are given by the set

$$ v_{T,g} = \max \left( \frac{Y_{c/g} r_{T,g}}{Y_{c/g} r_{T,g} + r_g} \right), $  \quad \gamma_{T} = \max \left( \frac{r_g}{Y_{c/g} r_{T,g} + r_g} \right). $$

(3.136)

3.5. Temporal Linkage: Growth and Network Model(s)

Lastly, before proceeding to the formulation of model equations and the subsequent analysis, we discuss the hypothetical regulation associated with the network input and output flux and the manifestation of this regulation via linkage of the network model to the abstracted growth model formulated above. We postulate that the key enzymes that catalyze the formation of $p_0$ and the consumption of $p_3^g$, $p_4^a$, and $p_8$ are functions of the growth phase of the culture. In other words, during the initial stages of the culture, the enzyme level is low. However, as the system moves toward balanced growth, in general, the catalytic enzyme levels also move towards a maximum level. Upon exhaustion of a system growth substrate, for example the carbon source, the level of catalytic enzymes is postulated to fall because of the lack of metabolic activity. In the present model system, an explicit formulation of the growth process as it relates to the aspartate network quickly, whereas, $\delta$ large implies the lag between an upper pathway metabolic perturbation and the corresponding response in the network input flux is long. In the present context, these perturbations are limited to alterations in the growth rate, i.e., perhaps changes in dilution rate in a continuous culture, or growth substrate concentration. We further postulate that

$$ r_{T,g} v_{T,g} \rightarrow \mu_{T,g} \Rightarrow V_{\text{flux}}^{p_0} \rightarrow 1 $$

$$ r_g v_g \rightarrow \mu_{T,g} \Rightarrow V_{\text{flux}}^{p_8} \rightarrow 1, \quad \gamma_j = 1, \quad j = p_3^a, p_4^a, p_8. $$

(3.139)

i.e., the maximum level of input and output flux into the network occurs under balanced growth conditions. This implies the proportionality constant(s) take the form

$$ \lambda = \frac{1}{\mu_{T,g}}, \quad \gamma_j = \frac{1}{\mu_{T,g}}, \quad j = p_3^a, p_4^a, p_8. $$

(3.140)

It follows that the flux control variable(s) are given functionally by

$$ V_{\text{flux}}^{p_0} = \frac{r_{T,g} v_{T,g} \left(1/\lambda\right)}{\mu_{T,g}^{\max}}, \quad V_{\text{flux}}^{p_8} = \frac{r_g v_g}{\mu_{T,g}^{\max}}, \quad j = p_3^a, p_4^a, p_8. $$

(3.141)

These variables modify the rate of input and output flux so as to mimic the changes in the levels and activity of the key enzymes that catalyze these fluxes. In this way we incorporate some sense of upper and lower pathway regulation as it relates to temporal external abiotic phase phenomena.

3.6. Complete Model System

The time evolution of the model system is governed by the following set of differential equations: Nonenzymatic state variables
Balance on network enzyme levels:

\[ \frac{d\rho_j}{dt} = r_{\rho_j} e_j - (r_g e_g + \beta_j) e_j + r_j^p, \quad j = 0, 1, 5, 6, 7 \]

\[ \frac{d\rho_{3j}}{dt} = r_{\rho_{3j}} u_{3j}(r_g e_g + \beta_j) e_j^3 + \rho_{3j}^0, \quad k = a, b \]

\[ \frac{d\rho_{4j}}{dt} = r_{\rho_{4j}} u_{4j}(r_g e_g + \beta_j) e_j^4 + r_{4j}^0. \]

Balance on network growth species:

\[ \frac{dS_g}{dt} = -\frac{1}{Y_{c/T}} r_{c/T, g} e_g T_g c \]

\[ \frac{dS_p}{dt} = r_{c/T, g} e_g - \frac{1}{Y_{c/p}} r_{c/g} e_g \]

\[ \frac{dP_{c/g}}{dt} = r_{c/T, g} e_g - (r_g e_g + \beta) e_g + r_g^p \]

\[ \frac{de_g}{dt} = r_g (r_g e_g + \beta) e_g + r_g^p \]

\[ \frac{dc}{dt} = r_g e_g c. \]

Balance on promoter inducer and transport species:

\[ \frac{dI_j}{dt} = -r_{T, j} c, \]

\[ \frac{dp^a_j}{dt} = r_{T, j} c + r_{g} e_g P_j, \]

\[ \frac{dP_j}{dt} = r_{d, p_j} c, \quad j = a, b \]

\[ \frac{dP_{c/g}}{dt} = r_{d, p_{c/g}} c, \]

\[ \frac{dP_{g}}{dt} = r_{d, p_{g}} c, \]

where \( r_j^p \) denotes the rate of constitutive enzyme synthesis defined as

\[
\begin{align*}
  r_j^p &= \begin{cases} 
    r_j^p & p_j = 0 \\
    r_j^p + \beta^p_j & p_j > 0,
  \end{cases} 
\end{align*}
\]

where \( \beta^p_j \) denotes the basal level of expression from the plasmid, i.e., \( \beta^p_j \) is a measurement of the extent of promoter leakage. The parameter \( \beta \) is the rate constant governing the first order decay of enzymatic machinery. The yield coefficient \( Y_{c/p} \) denotes the yield of biomass (g dw) per unit \( p_c \). Lastly, the maximum specific level of the \( j \)th key enzyme is readily determined from the \( e_j \) balance, given a few simplify assumptions. Firstly, we assume that enzyme synthesis is saturated with respect to inducer metabolite(s) as \( e_j \) approaches \( e_j^{\text{max}} \). Secondly, as \( e_j \rightarrow e_j^{\text{max}} \) the complete network \( u \)-variable governing the allocation of critical resources to the synthesis of \( e_j \) moves towards unity. Lastly, we assume that \( e_j \approx e_j^{\text{max}} \) when \( r_g e_g \approx r_g^p \). These assumptions imply

\[
\alpha_{e_j} - (r_g^{\text{max}} + \beta) e_j^{\text{max}} + r_c e_j \approx 0
\]
which can be easily solved for \( \varepsilon_j^{\text{max}} \) to yield

\[
\varepsilon_j^{\text{max}} = \frac{\mu_j^g + r_j^g}{\mu_j^g + \beta_j} \quad \forall j.
\]

(3.144)

\section*{4. ANALYSIS AND DISCUSSION}

Now that we have outlined the model framework, we begin the analysis portion of the development. Specifically, we review the experimental results of Stephanopoulos and Sinskey (Colón et al., 1995a, b; Jetten et al., 1995) and coworkers dealing with lysine and threonine overproduction in \textit{C. lactofermentum} ATCC 21799. This review is followed by examination of the model response to genetic perturbation. Specifically, we simulate the exact experimental alterations made by Stephanopoulos et al. and Sinskey et al. and contrast the model response with experimental observations. Our goal in this endeavor is to evaluate the cybernetic models ability to predict the manner in which pathway metabolic function reacts to alteration in local genetic structure.

\subsection*{4.1. Experimental Results of Stephanopoulos and Sinskey}

The focus of the series of papers authored by Stephanopoulos, Sinskey, and co-workers has been the construction of a threonine producing microbe using as a base a lysine overproducing strain. Specifically, the strain ATCC 21799(pM2) has incorporated into the chromosome a deregulated aspartokinase which is no longer sensitive to inhibition by lysine. This affords a microbe that has lysine titers (90 h, batch culture) of \( \sim 22 \text{ g/L} \). The objective of the experimental exercise is the conversion of the lysine producing ATCC 21799 (pM2) into a threonine overproducer. Given the scope of our investigation, we dispense with discussing the specifics of the genetic manipulations involved in the threonine overproducer construction. Rather, we focus upon the qualitative aspects involved, and moreover, examine the system given the previous branch point development. Experimental results reproduced from (Colón et al., 1995a) are shown in Table 2.

<table>
<thead>
<tr>
<th>Strain: Corynebacterium lactofermentum ATCC 21799</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Source: Glucose (80 g/L)</td>
</tr>
<tr>
<td>Sample Time: 90 h</td>
</tr>
</tbody>
</table>

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{C. glutamicum strain} & \textbf{Lysine} & \textbf{Threonine} & \textbf{Homoserine} & \textbf{Isoleucine} \\
\hline
ATCC 21799 (pM2) & 22.0 ± 1.0 & < 0.1 & < 0.1 & < 0.1 \\
ATCC 21799 (pJD4) & 4.3 ± 0.2 & 5.4 ± 0.2 & 2.0 ± 0.1 & 1.3 ± 0.1 \\
ATCC 21799 (pGC42) & No induction & 0.9 ± 0.1 & 5.6 ± 0.3 & 6.7 ± 0.3 & 1.0 ± 0.1 \\
& 1.5 \text{ \textmu}mol/100 ml & 0.8 ± 0.1 & 11.8 ± 0.6 & < 0.1 & 1.9 ± 0.2 \\
& 5 \text{ \textmu}mol/100 ml & 0.9 ± 0.1 & 9.3 ± 0.1 & < 0.1 & 0.9 ± 0.1 \\
\hline
\end{tabular}
\caption{Table of Experimental Results: ATCC 21799 (pM2) Conversion to Threonine Overproducer*}
\end{table}

* Reproduced, by permission, from G. Colon et al. (1995a).

al., 1994) have overexpressed a feedback insensitive homoserine dehydrogenase (\( \varepsilon_j^{\text{max}} \)) which did result in homoserine accumulation, however, large amounts of residual lysine also accumulated. Thus, the model predicted course of action did result in diversion of flux away from lysine, however, the increased flux toward threonine was stalled at homoserine rather than moving all the way to the target. Moreover, some flux still found its way to lysine. This implies, given the predictions of the branch point analysis, that the kinetic competition for \( p_j \) is not entirely in the favor of \( p_j \) formation. We examine this point subsequently.

The construct ATCC 21799 (pJD4) is designed to eliminate this situation by employing a push–pull strategy around the accumulating intermediate homoserine (\( p_j \)). Specifically, pJD4 contains the genes that encode for a feedback insensitive homoserine dehydrogenase (\( \varepsilon_j^{\text{max}} \)) as well as a wild-type homoserine kinase under their respective native promoters. The experimental results show homoserine (\( p_j \)) accumulation accompanied by residual lysine formation (\( p_j \)) in addition to threonine formation. Thus, the push–pull strategy does result in the formation of a substantial homoserine conversion; however, this configuration is clearly not optimal because large levels of lysine as well as homoserine still remain.

The construct ATCC 21799 (pGC42) approaches the problem of threonine formation from much the same perspective as pJD4 with the exception of the promotion. More exactly, pGC42 assumes homoserine kinase (\( \varepsilon_j^{\text{max}} \)) is under a tac promoter, whereas, homoserine dehydrogenase (\( \varepsilon_j^{\text{max}} \)) is under its native promoter. Interestingly, this configuration results in an unexplained drop in lysine formation for the no induction case and a nonlinear dependence upon the level of the promoter inducer IPTG. Specifically, the no
induction case is marked by low levels of lysine accumulation coupled with relatively high levels of threonine and homoserine accumulation. Upon induction with 1.5 μmol/100 ml of IPTG the bulk of the homoserine is converted to threonine and some lower pathway metabolites, specifically, isoleucine. Upon induction with 5 μmol/100 ml of IPTG the level of threonine accumulation falls well below the level resulting from induction at 1.5 μmol/100 ml of IPTG.

In what follows we examine the response of the model system to the genetic perturbations described above. Specifically, we fit the model parameters so as to describe the time evolution of ATCC 21799 (pM2) and then freeze the parameter set. This involves not only the description of biotic phase phenomena such as intermediate accumulation, but the prediction of abiotic phase evolution as well. We make a special point in this regard because of our model structure. More exactly, we have assumed the input and output flux of the network model to be directly proportional to the glucose uptake rate and system growth rate, respectively, where the input flux is delayed by δ h. This follows from the postulate that upper metabolic activity drains in the direction of the aspartate family δ hours after growth has ceased. Thus, description of the external operating environment is key to the temporal aspect of the model prediction. This perspective is much the same as the penicillin V biosynthetic network model formulated and evaluated in the next paper, i.e., a simple abstracted growth model drives the temporal aspects of the aspartate network model so as to describe both the external and internal culture time evolution. Before moving to the evaluation of the genetic perturbations we take a few a moments to discuss the cybernetic significance of the overexpression of existing pathway enzymes given a frozen parameter set.

The cybernetic framework is a goal-oriented methodology that assume a microbe regulates the synthesis and activity of key enzymes so as to direct its nutritional state toward an objective. In the present context, given the model formulation, the local objective of the aspartate family network is postulated to be the maximization of the levels of lysine, methionine and isoleucine. Thus, by fitting the base structure, pJD4 and pGC42. The algorithm is as follows: Firstly, we determine a model parameter set such that pM2 evolution is captured. This includes both macroscopic external variables such as growth substrate and biomass levels in addition to specific levels of metabolic intermediates. The experimental results of Stephanopoulos et al. and Sinskey et al. consists of only a single point taken at t = 90 h. Unfortunately, this is the extent of the experimental observations at hand, thus, our object is to compare the model predicted level of metabolic intermediates at t = 90 h with experimental values.

The microbes were aerobically cultured at 30°C (250 rpm) in 500 ml baffled flasks (100 ml of C. glutamicum) minimal media adapted from von der Osten and Sinskey (von der Osten and Sinskey, 1989) and Kiss (Kiss, 1991) which contained kanamycin (50 mg/L) and ampicillin (50 mg/L) and glucose as a carbon source (80 g/L). It was reported that all the glucose was consumed by t = 70 h yielding a final biomass titer of ~1.8 (gDw/L). As an additional input we employ the flux analysis results of Stephanopoulos and Vallino (Vallino and Stephanopoulos, 1993) for an organism similar to pM2 producing lysine to get a handle on the parametric nature of glucose uptake. From this data as well as the experimental information, we are able to determine system parameters such that abstracted growth model is consistent with experimental observations. The maximum specific growth rate from the precursor pool p was predicted to be μ_{max} = 0.75 h^{-1} and the biomass yield Y_{cx}=0.0219 (gDw/g). The model predicted glucose uptake rate during balanced growth (14.75 mmol/L·h) compares well with that predicted by the flux analysis.

(~ 15 mmol/L-h.) Using these parameters, the model simulation of the abiotic state of the system is shown in Fig. 3. Note, it was reported that the growth characteristics of each of the constructs was similar. Thus, the growth performance of the microbe is not qualitatively effected by the presence of the plasmid. This implies the same growth model, i.e., parametrically identical, can be used throughout our study.

The construction of the base strain ATCC 21799(pM2) (which also carries a shuttle vector encoding for antibiotic resistance) results from the incorporation of a lysine feedback insensitive aspartate kinase (e_0) into the chromosome of ATCC 21799 (Jetten et al., 1995). This is represented within the model framework by assuming \( V_{e_0, p_3, a_r} \gg 1 \). This is all that is required for the description of a genetic change incorporated directly into the chromosome because, unlike plasmid borne alterations, there does not exist multiple enzyme expression sources. In addition to the experimental results discussed above, we again turn to the flux analysis of Stephanopoulos and Vallino (Vallino and Stephanopoulos, 1993) to get a handle on the input flux into the aspartate network. The simulation results are shown in Fig. 4 where the abiotic phase lysine level is plotted versus time. Notice the onset of the accumulation regime approximately at \( t = 65 \) h followed by an inflection near \( t = 75 \) h, and eventually, accumulation stoppage. These temporal regions clearly stem from the influence of the macroscopic growth model and the assumption of upper pathway drainage. Near \( t = 65 \) h the uptake and growth rate(s) slow and eventually drop to zero which implies the aspartate family output flux toward biomass formation, because we have assumed a direct proportionality with the growth rate, decays to zero.

The construct pJD4 assumes the gene for a threonine feedback insensitive homoserine dehydrogenase (e_2) and a wild-type homoserine kinase (e_3) are incorporated, under their respective native promoters, on a plasmid inserted into the base pM2 construct (in place of the shuttle vector.) The assumption of a feedback insensitive \( e_2 \) implies \( \psi_{e_2, p_3, a_r} \gg 1 \). Because both enzymes are native to ATCC 21799 (pM2), there exists multiple sources of enzyme expression, i.e., from the plasmid or the chromosome. Note, in the case of \( e_2 \), feedback insensitivity to the level of threonine is not a

Experimental Value: 22 (g/L) ± 1.0

Predicted Value: 22.75 (g/L)

FIG. 4. Simulations results for abiotic lysine level of pM2.

This results in intermediate accumulation because we assume the upper pathway metabolic drainage occurs for \( \delta h \) after the cessation of glucose uptake. The inflection indicates a point where the production of lysine is becoming substrate limited, i.e., the influx into the \( p_2 \) branch point slows and eventually stops. Note the predicted lysine level agrees well with experimental results. Moreover, although not shown, the lower pathway results, i.e., for the levels of the various other metabolic intermediates sampled by Stephanopoulos et al., also can be shown to agree within experimental error. Additionally, the input flux into the aspartate network, denoted by \( R_0 V_{\text{flux}} \), is in good agreement with the predicted flux analysis value. In particular, the simulation predicts (during balanced growth) \( R_0 V_{\text{flux}} = 4.23 \) mmol/h-L, whereas, Stephanopoulos and Vallino predict 2.95 mmol/h-L.

At this point we freeze the parameter set used in the pM2 simulation and examine the ramifications of the genetic alterations.

The construct pJD4 assumes the gene for a threonine feedback insensitive homoserine dehydrogenase (e_2) and a wild-type homoserine kinase (e_3) are incorporated, under their respective native promoters, on a plasmid inserted into the base pM2 construct (in place of the shuttle vector.) The assumption of a feedback insensitive \( e_2 \) implies \( \psi_{e_2, p_3, a_r} \gg 1 \). Because both enzymes are native to ATCC 21799 (pM2), there exists multiple sources of enzyme expression, i.e., from the plasmid or the chromosome. Note, in the case of \( e_2 \), feedback insensitivity to the level of threonine is not a

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Note: The text contains a series of mathematical and scientific terms that are typical in metabolic engineering research, focusing on the simulation and analysis of microbial growth and metabolic pathways. The figures and equations mentioned in the text are illustrative of the simulation results and the experimental validations.
feature of the wild type enzyme. The gene that encodes for a feedback insensitive $e_{b}^{3}$ was isolated from a mutant strain and incorporated onto pJD4. The importance of such a detail follows from the derivation of the global control that regulates the choice of synthesis source. In particular, we have assumed the competition that regulates synthesis source is substitutable, i.e., parallel routes competing to produce a common product. Within the current context, these parallel routes consist of the chromosomal and plasmid expression sources of $e_{b}^{3}$. However, the gene encoded on the plasmid yields a feedback insensitive $e_{b}^{3}$, whereas, the chromosomal source yields a wild type $e_{b}^{3}$. These two enzymes are clearly not identical, thus, assumption of substitutable competition is not strictly valid. We, however, acquiesce from such issues and assume the chromosomal route is slow in comparison to the plasmid route. Thus, in a short period of time the majority of the $e_{b}^{3}$ stems from the plasmid expression route and the ratio of feedback insensitive $e_{b}^{3}$ to wild type $e_{b}^{3}$ is strongly in favor of the former. This understanding allows us to treat these cases using the standard assumptions described in the first paper of this series. Of course, $e_{b}^{3}$ overexpression can be described using the standard development. Accordingly, to account for the possibility of multiple expression sources for the key enzymes $e_{b}^{3}$ and $e_{b}^{3}$ the synthesis source terms in the $e_{b}^{3}$ and $e_{b}^{3}$ balances, i.e., $r_{b}^{3}U_{gb}$ and $r_{b}^{3}U_{gb}$ must be replaced by

$$\begin{align*}
r_{b}^{3}U_{gb}^{e} + r_{b}^{3}U_{gb}^{p}, \quad j = 2, 3, \quad (4.1)
\end{align*}$$

where the second expression term $r_{b}^{3}U_{gb}^{e}$ denotes the expression of enzyme $e_{b}^{3}$ from the plasmid. The control variables $U_{gb}^{e}$, $U_{gb}^{p}$, $k = g, p$ denote the global cybernetic variables that regulate the choice of synthesis source of the enzyme $e_{b}^{3}$. The superscript $e$ and $p$ denote genome and plasmid, respectively. The form of the global control variables was derived previously so we dispense with a formal derivation. In short, we have postulated that the multiple expression sources of enzyme $e_{b}^{3}$ are substitutable, thus, the global control variable that regulates the choice of expression source take the form

$$\begin{align*}
U_{gb}^{e} = \frac{r_{b}^{3}U_{gb}^{e}}{r_{b}^{3}U_{gb}^{e} + r_{b}^{3}U_{gb}^{p}}, \quad U_{gb}^{p} = \frac{r_{b}^{3}U_{gb}^{p}}{r_{b}^{3}U_{gb}^{e} + r_{b}^{3}U_{gb}^{p}}, \quad j = 2, 3, \quad (4.2)
\end{align*}$$

where the rate of enzyme expression from the plasmid takes the form

$$\begin{align*}
r_{b}^{3}U_{gb}^{p} = \frac{p_{b}^{3}}{K_{b}^{3} + p_{b}^{3}}, \quad K_{b}^{3} = p_{b}^{3} + p_{b}^{3}, \quad (4.3)
\end{align*}$$

The parameter $k$ denotes the sensitivity of macroscopic enzyme expression to changes in copy number. Consistent with the copy number heuristic of Shuler (Schuler, 1992) we assume $k < 1$. The model prediction of the overexpression of a feedback insensitive $e_{b}^{3}$ and a wild-type $e_{b}^{3}$ versus experiment is shown in Fig. 5. The simulated overexpression of $e_{b}^{3}$...
and $e_b^4$ results in an $\sim 7$-fold increase in the level of $e_b^3$ and an $\sim 6$-fold increase in the level of $e_b^3$. These results are consistent with the experimental findings of Stephanopoulos and co-workers (Colón et al., 1995a) and are tabulated in Table 3.

The model predicted response agrees well with that observed experimentally. The various temporal regions of accumulation as well as the effect of system feedback interactions are clearly visible from the simulation. The onset of accumulation occurs as the system growth rate decays to zero consistent with the discussion above. The low level of isoleucine indicates flow into the lower pathway below $y = 0$ consistent with the experimental findings of Stephanopoulos and co-workers (Colon et al., 1995a) and are tabulated in Table 3. The observations are borne out through simulation of the network model. Moreover, these findings clearly illustrate that redirection of the flux away from lysine formation toward threonine is composed of two aspects, namely, the diversion of flux at the $p_2$ branch point and the elimination of homoserine accumulation. As a thought experiment (parameter set slightly different than pM2) to test our hypothesis that a stronger promoter governing feedback insensitive $e_b^4$ expression could eliminate lysine accumulation, we consider the overexpression of a feedback insensitive homoserine dehydrogenase under artificial and native promoter(s). To account for the possibility of multiple sources of $e_b^4$ we replace the rate of enzyme synthesis in the $e_b^4$ balance with Eq. (4.1) where the rate of expression from the plasmid is given by the form

$$r_{e_b}^4 = \frac{p_L e_b^4}{K_{e_b}^P + p_L e_b^4},$$

where $\alpha_b^P$, $K_{e_b}^P$ denote the rate and saturation constants that govern the expression from the plasmid of $e_b^4$. The rate constant $\alpha_b^P$ takes the power law form given above and the maximum rate of expression from a single plasmid, i.e., our measure of promoter strength is left as a parameter in the artificial case. Additionally, in the case of artificial amplification, the levels of $e_b^4$, $j = 2, 3$ are increased, and more importantly, the activity of these enzymes increases dramatically affording $e_b^4$ an increased share of the precursor metabolite $p_2$. However, some lysine formation is observed even in the amplified pathway. This indicates that $e_b^4$ is not being overexpressed strongly enough to completely pull $p_2$ away from $p_4$ formation, and moreover, a sizable portion of the flux that has been diverted is wasted in the accumulation of homoserine ($p_3^b$). The observations are consistent with the experimental findings of Stephanopoulos and coworkers (Colon et al., 1995a) and are tabulated in Table 3.

| TABLE 3 |
| Level of Amplification of $e_b^4$ and $e_b^3$ for Construct pJD4, pGC42 as Compared with pM2 at $t = 50$ h. |

<table>
<thead>
<tr>
<th>Simulated enzyme level and activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construct</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>pM2</td>
</tr>
<tr>
<td>pJD4</td>
</tr>
<tr>
<td>pGC42</td>
</tr>
<tr>
<td>No Induction</td>
</tr>
<tr>
<td>1.5 $\mu$mol/100 ml</td>
</tr>
<tr>
<td>5.0 $\mu$mol/100 ml</td>
</tr>
</tbody>
</table>

Note. In the case of pGC42 induction is initiated at $t = 30$ h.
promoted, the inducer (5 μmol/100 ml) is introduced into the media at \( t = 30 \) h. The system response to overexpression both in terms of intermediate level as well as enzymatic level and activity are tabulated in Tables 4 and 5, respectively. Consider the native promotion case. The level of \( e_2^b \) increases \( \sim 3 \) fold whereas the activity increases dramatically. In contrast, the level and activity of \( e_2^b \) decrease significantly. This allows \( e_2^b \) to capture a larger share of \( p_2 \) and the level of lysine falls accordingly. Note, however, there is still a substantial lysine accumulation. This implies, even though \( e_2^b \) levels and activity have been increased by at least 3-fold, the resistance to flux alteration still affords lysine accumulation. The artificial promoter case for a promoter strength equivalent to native (hom\(^{\text{N}}\)-(\( p_1 \)) case) is nearly the same as wild type. Increasing promoter strength by a factor of 2 yields drastically different results. At this level of promotion, the flux through the \( p_2 \) branch point is almost preferentially drawn towards the formation of homoserine, but not threonine, hence, the need for the push promoter strength to almost preferentially draw flux toward threonine and away from lysine. Thus, the model system clearly predicts the failure of a simple deregulation pull approach to redirect flux toward threonine away from lysine.

The construct pGC42 assumes the gene that encodes for a threonine feedback insensitive \( e_2^b \) and the gene that encodes for a wild-type \( e_2^b \) are under separate promoters. Specifically, \( e_2^b \) is under its native promoter, whereas, \( e_2^b \) is under an artificial promoter (\( tac \)). To account for the multiple sources of key enzyme \( e_2^b \) and \( e_2^b \) we replace the enzyme synthesis terms in the \( e_2^b \) and \( e_2^b \) balances with those shown in Eq. (4.1). The functional forms of the global control variables \( U_{g}^k \) \( \kappa = g, p \) where superscript \( g \) and \( p \) denote genome and plasmid, respectively, are identical to those given previously. The change in promoter configuration is reflected in the model configuration by the form of the rate of enzyme expression from the plasmid, i.e., \( r_{p}^b \). The overexpression of the enzyme \( e_2^b \) remains under its native promoter which implies \( r_{p}^b \) is identical to the previous case. The overexpression of \( e_2^b \) is now assumed to be under an artificial promoter which implies \( r_{p}^b \) is of the form

\[
r_{p_2}^b = \frac{\eta_{p, e}^b \cdot \frac{b}{b}}{K_e^p + b \cdot e},
\]

where \( \eta_{p, e}^b \), \( K_e^p \), denote the rate and saturation constants that govern the overexpression of \( e_2^b \). The rate constant \( \eta_{p, e}^b \) is given by the power law form shown above and the maximum rate of \( e_2^b \) expression from a single plasmid is an order of magnitude greater than the maximum rate of wild type \( e_2^b \) expression, i.e.,

\[
\eta_{p, e}(e) = \frac{\eta_{p, e}(e)}{\eta_{p, e}(0)} = 10
\]

The model simulations of pGC42 under three different expression loads are shown in Figs. 6–8. The results in terms of enzyme level enhancement are shown in Table 3. Note the latter set of results is qualitatively consistent with the experimental development of Stephanopoulos et al. (Colón et al., 1995a).

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**TABLE 4**

Enzymatic Response to Expression of Feedback Resistant Homoserine Dehydrogenase as a Function of Promoter Type and Promoter Strength at \( t = 50 \) h.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Lysine</th>
<th>Homoserine</th>
<th>Threonine</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>24.724</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>hom(^{\text{N}})-N</td>
<td>4.5378</td>
<td>4.0687</td>
<td>3.9377</td>
</tr>
<tr>
<td>hom(^{\text{N}})-(( p_1 ))</td>
<td>24.6812</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>hom(^{\text{N}})-(( p_2 ))</td>
<td>1.661</td>
<td>6.5045</td>
<td>3.7539</td>
</tr>
</tbody>
</table>

Note. When applicable inducer (5 μmol/100 ml) is introduced at \( t = 30 \) h.
The pGC42 experimental data has an interesting feature associated with the no induction case, namely, an unexplained reduction in the level of lysine and moreover a nonlinear response to the levels of IPTG. Colon et al. does not give a rationale as to why this might be so, thus, we employ the model framework to explain these experimental observations. We postulate the no induction response and the nonlinear network response to induction levels follows as a consequence of a cooperation between the artificial and natural promoters. This hypothesis follows straightaway from an understanding of the behavior of flexible nodes presented in the preceding paper of this series. Intuitively, because $e_{2}^{b}$ overexpression is under the control of its respective native promoter, we expect the lysine level in the no induction case to be identical with pJD4. However, this is found not to be the case. Rather, it is observed that lysine levels drop dramatically in pGC42. The model system predicts that the drop in lysine level follows as a consequence of a postulated enhancement of the $e_{2}^{b}$ native promoter strength. Specifically, it was found by simulation that the native promoter strength of $e_{2}^{b}$ must increase by a factor of 2.5 to account for the decrease in lysine levels. Given this adjustment, the model predicted system response matches well the experimental observations. Note a biological basis for this hypothesis is currently under investigation.
Another obvious possibility for the unexplained decrease in lysine level in construct pGC42 is basal expression stemming from a “leaky” artificial promoter. However, hom+ (under its native promoter) is cloned in front of homoserine kinase, thus, leaky expression of the kinase should have no obvious effect on lysine levels, i.e., should not drastically impact $e_3^b$ levels. Thus, it would seem that this explanation is unlikely.

The nonlinear response with respect to induction level can also be explained given the cooperation hypothesis. We postulate the rate of expression of $e_3^b$ under its native promoter is influenced by the level of induction of $e_3^b$ over-expression. It is predicted from simulation, the maximum rate of expression of $e_3^b$ must pass through a maximum near the 1.5 $\mu$mol/100 ml induction level and then decrease as the inducer concentration increases. Specifically, the native promoter strength must be increased by a factor of 1.27 over the no induction case to capture to the experimental promoter strength must be increased by a factor of 1.27 over the no induction case to capture to the experimental observations. Given these adjustments, the model predicted response(s) shown in Figs. 7 and 8 agrees well with experimental observations.

The cooperation mechanism can be brought into the current framework through the reformulation of the maximum rate of enzyme expression from a single plasmid. Specifically, the current functional power law form could be modified such that

$$
\frac{\alpha_i^p}{\alpha_i^p_{no}} = \frac{f(X)(N)^k}{f(X)} + b
$$

where $f(X)$ is a function that describes the sensitivity of the promotion of $e_3$ to culture conditions, such as, temperature, specific metabolite levels or operating environment. A systematic formulation of $f(X)$ to date, however, has not been undertaken.

5. CONCLUSIONS

Using the modular approach, we formulated a cybernetic model that describes the time evolution of the aspartate family of amino acids. The model framework was subjected to genetic perturbations, as outlined by Stephanopoulos et al. and Sinskey et al. and the network model responses were compared to experimental observations. It was found that an appropriately modified base model system predicted, for the most part within experimental error, the experimentally observed local network response under a number of different perturbations.

The deeper objective of this development is to evaluate the cybernetic framework’s ability to describe the manner in which a metabolic network responds locally to genetic alteration. This feature has direct bearing on the utility of the metabolic engineering development outlined in briefly here and in more detail in (Varner and Ramkrishna, 1998a, b). The implication of this set of results is far reaching in terms of the conceptual applicability of a cybernetic metabolic engineering approach. Primarily, it lends credence to the assumption that local genetic alterations do not alter the presumed local network objective, and moreover, that a recombinant system is still optimal with respect to resource allocation albeit to an expanded or contracted set of alternatives. Certainly, the quality of the simulation results presented could be improved by paying more careful attention to the kinetic forms of the rates involved in the pathway and by formulating more sophisticated representations of the effects of metabolite feedback. Additionally, the availability of more experimental information as to the wild-type state and the response of the system to perturbation could definitely aid in improving the quality of prediction. However, these issues aside, the proximity of model prediction and experimental observation is sufficiently close to warrant the suggestion that the founding postulates of the framework put forth have conceptual merit.

REFERENCES


