Cybernetic Modeling and Regulation of Metabolic Pathways in Multiple Steady States of Hybridoma Cells

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Hybridoma cells utilize a pair of complementary and partially substitutable substrates, glucose and glutamine, for growth. It has been shown that cellular metabolism shifts under different culture conditions. When those cultures at different metabolic states are switched to a continuous mode, they reach different steady states under the same operating conditions. A cybernetic model was constructed to describe the complementary and partial substitutable nature of substrate utilization. The model successfully predicted the metabolic shift and multiple steady-state behavior. The results are consistent with the experimental observation that the history of the culture affects the resulting steady state.

Introduction

Mammalian cell culture processes are widely used in the manufacture of many recombinant therapeutic proteins, viral vaccines, and vectors for gene therapy. The growth medium used for their cultivation is usually rather complex. A typical cell culture medium consists of glucose and glutamine as the main sources for carbon and energy (*1*−*3*) and also other amino acids, vitamins, and nucleotides. Often the medium is also supplemented with animal serum or other supplements.

Glucose metabolism is linked to glutamine metabolism via the TCA cycle. The uptake rate of glutamine far exceeds what is needed for its incorporation into biomass (as cellular protein or nucleotides) and protein products. The majority of glutamine taken up by cells enters the TCA cycle via glutamate and α-ketoglutarate. Glutamine provides the carbon skeleton and amino groups for cellular materials or is oxidized to provide energy. From the perspective of energetics, cells can derive their energy needs from different combinations of glucose and glutamine (*4*, *5*). They are therefore substitutable (*6*). For the purpose of supplying cells with building blocks of biomass, glucose and glutamine are both required nutrients. They are thus also complementary to each other. From a modeling perspective, glucose and glutamine are partially substitutable and complementary substrates.

The metabolism of glucose is greatly affected by its own concentration (*7*−*9*). At high glucose levels, it is consumed at a faster specific rate; about 70−95% of the glucose consumed is converted to lactate. At low glucose concentrations, the consumption rate is lower and a larger proportion is completely oxidized to CO_2 (*10*). Using a fed-batch reactor to manipulate glucose at a low level (as compared to a typical batch culture), cell metabolism can be altered to a state with substantially reduced lactate production (*11*−*14*). Once the low lactate producing metabolic state is achieved, the culture can then be switched to a continuous mode and allowed to reach a steady state while maintaining the low lactate producing metabolism. The concentrations of cells and antibody were substantially higher than a control culture that was initiated from a batch culture without first altering cellular metabolism (*15*). The lactate and other metabolite concentrations were also substantially reduced as compared to the control culture. Using the same dilution rate and feed medium, the control culture and the metabolically shifted culture (derived from a fed-batch culture) reached different steady states. The only difference was that the initial conditions or the paths leading to the two steady states were different. These results demonstrate steady-state multiplicity.

It is desirable to develop a model that is suitable for describing the partially substitutable and complementary nature of glucose and glutamine and is capable of predicting different culture conditions leading to different steady states. Our approach to this problem is to apply a cybernetic model. A salient feature of the cybernetic model is its treatment of metabolic switch or regulation. When faced with environmental changes, cells frequently have more than one possible response in their metabolic machinery. The cybernetic model assumes that the cells' response is based on economic principles by maximizing its return (the speed of obtaining the desired results) on its investment (the cost associated with making new enzymes or other metabolic machinery). As in metabolic reaction models a cybernetic model starts with a simplified reaction pathway or network based on biochemical knowledge. It postulates that the cell must invest a fixed amount of resources for the synthesis of enzymes and regulate their activities in an optimal manner. For every branched or converged junction in a metabolic pathway, it assumes that cells switch their metabolism in response to changes in their environment in a manner consistent

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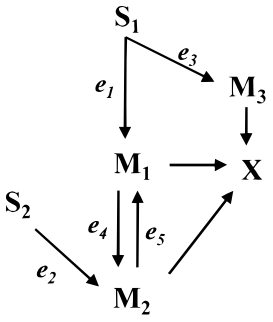

Figure 1. A model system for mammalian cell metabolism that includes glucose (S_1), glutamine (S_2), intracellular precursors (M_i), cell density (X), and specific level of the i key enzyme (e_i).

with optimal goals. In grossly simplified pathways, the optimal goal may be global, such as maximizing biomass (*16*).

We postulate that the adaptation of the mammalian cell to a changing environment can be described using cybernetic principles, in which the physiology operates to optimally satisfy nutritional objectives. In this paper, we use the cybernetic approach to predict and possibly explain the multiplicity of steady states observed experimentally. The model will provide valuable information about the regulation of metabolic pathways involved in the alteration of cell metabolism.

Model Development

To explore the feasibility of using a cybernetic model to predict the multiple steady-state behavior in mammalian cell culture, we considered a simplified metabolic reaction network involving only glucose and glutamine as shown in Figure 1. It is assumed that all other amino acids, except glutamine, are supplied in sufficient concentrations to support growth and product formation, but not in excess to engage in energy metabolism. Their effect on cell growth is thus neglected. Glucose, S_1, is metabolized as a carbon-energy source and its carbon flux can be directed to two paths, the pentose pathway and glycolysis, giving rise to two intermediates, M_1 and M_3. M_3 represents the glucose-derived building blocks of cellular materials such as riboses; M_1 represents a pool which includes pyruvate and lactate. Pyruvate can serve as a precursor for incorporation into biomass, X, via acetyl-CoA and fatty acids, or it can enter the TCA cycle. As the rate of glycolysis is usually faster than the maximum rate of utilization of glycolitic intermediates, most glucose is metabolized to lactate, which is reflected by the accumulation of M_1.

Glutamine, S_2, is a nitrogen as well as carbon and energy source. Glutamine is partially oxidized to glutamate after releasing ammonia. Glutamate can be deaminated to become M_2, which does not contain nitrogen atoms, or provide nitrogen to form nonessential amino acids from M_2 if they are in short supply. Our motivation in this work is to produce a preliminary model that has the broad features of the regulatory phenomena observed experimentally, so a suitably simplified formulation was deemed appropriate. Thus, M_2 represents a lumped pool of intermediates in the TCA cycle. M_1 and M_2 are interchangeable through the TCA cycle. After passing through the TCA cycle, the glutamine carbons can contribute to the formation of biomass through M_1 or M_2.

The rate of biomass synthesis is assumed to be dependent on the concentrations of the precursor pools, M_1, M_3, and M_2. The partially substitutable and complementary nature of S_1 and S_2 are clearly seen in the synthesis of these three intermediates. S_1 is utilized for two possible alternatives: the synthesis of M_1 and M_3, both essential for cell growth. The two pathways compete to utilize the same substrate, S_1, and are therefore considered as complementary. The formation of M_1 is possible either via S_1 or via M_2. Thus a given synthesis rate of M_1 can be achieved by a number of linear combinations of fluxes in each branch. Similarly, the formation of M_2 can be achieved from either S_2 or M_1. These are substitutable processes in which both alternatives compete to synthesize the same product. All these competitive alternatives potentially respond to a regulatory mechanism that results in different flux distributions.

Facing changes in the environment, cells respond by altering the internal structure using preferential metabolic pathways. The shift between different pathways is controlled by internal regulation capable of coordinating the synthetic rates and the activities of many enzymes. The regulation mechanism of these enzymes can thus lead to different growth dynamics.

Process Equations and Kinetics

Following the framework shown in Figure 1, the consumption rate (r_i) of the carbon source (S_j) to form intermediates (M_i) catalyzed by enzyme (e_i) is assumed to follow Monod-type kinetics as shown below:

$$r_i = r_i^{\max} \frac{S_j}{S_j + K_i}\left(\frac{e_i}{e_i^{\max}}\right) \quad (i, j) = (1, 1), (3, 1), (2, 2)$$

where $r_i^{\max}$ and K_i are the maximum rate and saturation constant respectively and $e_i^{\max}$ denotes the maximum level of enzyme e_i. It is assumed that the synthetic rate of enzyme e_i (denoted as r_{ei}) is dependent on its substrate, S_j, following saturation-type kinetics:

$$r_{ei} = r_{ei}^{\max} \frac{S_j}{S_j + K_{ei}} \quad (i, j) = (1, 1), (2, 2), (3, 1)$$

where r_{ei}^{max} and K_{ei} are the maximum rate and saturation constant, respectively, governing the synthesis of e_i.

The intermediates M_1 and M_2 are utilized to synthesize each other by reactions catalyzed by e_4 and e_5 respectively:

$$r_i = r_i^{\max} \frac{M_h}{M_h + K_i}\left(\frac{e_i}{e_i^{\max}}\right) \quad (i, h) = (4, 1), (5, 2)$$

where $r_i^{\max}$ and K_i are the maximum rate and saturation constants. All three intermediates are essential for cell growth, and the growth rate can be described by a multiplicative Monod-type function of M_1, M_2, and M_3 (*17*). It is assumed that the enzymes for biomass formation are always present at the maximum level. The growth rate is given by the rate expression

$$r_g = r_g^{\max}\left(\frac{M_1}{M_1 + K_{g,M_1}}\right)\left(\frac{M_2}{M_2 + K_{g,M_2}}\right)\left(\frac{M_3}{M_3 + K_{g,M_3}}\right)$$

where $r_g^{\max}$ and K_{g,M_i} are the maximum growth rate and growth saturation constant, respectively.

Metabolic Regulation

Metabolic regulation, through which cells respond to changing environment, is proposed to be effectuated by cybernetic variables for complementary and substitutable processes as proposed by Straight and Ramkrishna (*18, 19*). One set of cybernetic variables, u_i, regulates the rate of enzyme synthesis; the other, v_i, regulates the activity of the enzyme. The regulation is assumed to be based on

Table 1. Model Parameter Values

parameter	value	parameter	value	parameter	value
r_1^{max}	0.05 g/gdw-h	$r_{e_5}^{max}$	0.001 g/gdw-h	b_4	0.1 gdw/gdw-h
r_2^{max}	0.04 g/gdw-h	K_{e_1}	0.001 g/L	b_5	0.05 gdw/gdw-h
r_3^{max}	0.03 g/gdw-h	K_{e_2}	0.0001 g/L	Y_1	0.9 gM_1/gS_1
r_4^{max}	0.04 g/gdw-h	K_{e_3}	0.001 g/L	Y_2	0.9 gM_2/gS_2
r_5^{max}	0.03 g/gdw-h	K_{e_4}	0.00001 g/L	Y_3	0.8 gM_1/gS_3
K_1	0.001 g/L	K_{e_5}	1×10^{-5} g/L	Y_4	0.6 gM_1/gM_2
K_2	0.001g/L	$r_{e_1}^*$	1×10^{-6} g/gdw−h	Y_5	0.86 gM_2/gM_1
K_3	0.001 g/L	$r_{e_2}^*$	1×10^{-6} g/gdw−h	r_g^{max}	0.0575 gdw/gdw-h
K_4	0.01 g/gdw	$r_{e_3}^*$	1×10^{-6} g/gdw−h	K_{gM_1}	0.0005 g/gdw
K_5	0.0001 g/gdw	$r_{e_4}^*$	1×10^{-6} g/gdw−h	K_{gM_2}	0.0005 g/gdw
$r_{e_1}^{max}$	0. 001 g/gdw-h	$r_{e_5}^*$	1×10^{-6} g/gdw−h	K_{gM_3}	0.0005 g/gdw
$r_{e_2}^{max}$	0.0005 g/gdw-h	b_1	0.05 gdw/gdw-h	Y_{m_1x}	0.7 gM_1/gdw
$r_{e_3}^{max}$	0.001 g/gdw-h	b_2	0.1 gdw/gdw-h	Y_{m_2x}	0.99 gM_2/gdw
$r_{e_4}^{max}$	0.0005 g/gdw-h	b_3	0.1 gdw/gdw-h	Y_{m_3x}	0.1 gM_3/gdw

the principle of optimal allocation of cellular resources to achieve an objective function.

Straight and Ramkrishna (1994a) propose that the objective function of the complementary competition (the formation of M_1 and M_3) is to maximize the mathematical product of the levels of the end product. The cybernetic variables can be determined from the matching law equation with the functional forms shown below:

$$u_i^c = \frac{r_i/M_h}{\sum r_i/M_h} \quad v_i^c = \frac{r_i/M_h}{\max(r_i/M_h)} \quad (i, h) = (1, 1), (3, 3)$$

The synthesis and activity of the key enzymes e_1 and e_3 are modulated by the cybernetic variables for complementary processes as $r_{ei}u_i^c$ and $r_iv_i^c$, respectively. The regulation promotes the utilization of the pathway that is most limiting. This is typical of a rigid node which is marked by a direct regulatory interaction on a branch point of a pathway at which one acts as a positive effector of the opposite branch reaction. The regulation prevents the accumulation of a relative excess of either M_1 or M_3. If M_1 is large then the return on investment as measured by r_1/M_1 becomes small and the corresponding rate of formation of M_1 is reduced as a result of a lower level of e_1. The same regulation is applied to the other divergent pathway for the formation of M_3.

For a substitutable process, the substitutable (the formation of M_1 from S_1 and M_2, and the formation of M_2 from S_2 and M_1) objective is to *maximize* the end product. This objective implies the cybernetic variables given by

$$u_i^s = \frac{r_i}{\sum r_i} \quad v_i^s = \frac{r_i}{\max(r_i)} \quad i = 1, 2, 4, 5$$

The regulated synthesis and activity of the key enzymes are given by $r_{ei}u_i^s$ and rv_{ii}^s.

Enzyme e_1 forms part of both complementary and substitutable processes. The synthesis and activity of this enzyme are thus $r_{ei}u_i^s\,u_i^c$ and $r_iv_i^s\,v_i^c$, respectively.

Model System

Two sets of balance equations describe the process: one for the substrate and cell concentration in the reactor, and the other for intracellular levels of enzymes and precursors.

Balance on substrates and biomass in the reactor

$$\frac{dS_1}{dt} = -(r_1v_1^s\,v_1^c + r_3v_3^c)X + D(S_1^f - S_1)$$

$$\frac{dX}{dt} = (r_g - D)X$$

$$\frac{dS_2}{dt} = -r_2v_2^sX + D(S_2^f - S_2)$$

Balance on intracellular precursors

$$\frac{dM_1}{dt} = Y_1r_1v_1^s\,v_1^c + Y_4r_5v_5^s - Y_{m_1x}r_g - r_gM_1 - r_4$$

$$\frac{dM_2}{dt} = Y_2r_2v_2^s + Y_5r_4v_4^s - Y_{m_2x}r_g - r_5v_5^s - r_gM_2$$

$$\frac{dM_3}{dt} = Y_3r_3v_3^c - Y_{m_3x}r_g - r_gM_3$$

Balances on enzyme levels

$$\frac{de_1}{dt} = r_{e_1}^* + r_{e_1}u_1^s\,u_1^c - (r_g + b_1)e_1$$

$$\frac{de_2}{dt} = r_{e_2}^* + r_{e_2}u_2^c - (r_g + b_2)e_2$$

$$\frac{de_3}{dt} = r_{e_3}^* + r_{e_3}u_3^c - (r_g + b_3)e_3$$

$$\frac{de_4}{dt} = r_{e_4}^* + r_{e_4}u_4^s - (r_g + b_4)e_4$$

$$\frac{de_5}{dt} = r_{e_5}^* + r_{e_5}u_5^s - (r_g + b_5)e_5$$

Where D is the dilution rate; S_j^f is the substrate concentration in the feed; Y_i is the yield coefficients; r_{ei}^* is the constitutive synthesis rate of the enzyme e_i, and b_i is the degradation rate constant of the enzyme. The term r_ge_i represents the dilution in the concentration of e_i due to cell growth. The precursor pools are also diluted through expansion of the biomass, r_gM_i. The maximum specific level of the key enzymes is estimated from the e_i balances:

$$r_{ei}^{max} - (r_i^{max} + b_i)e_i^{max} + r_{ei}^* = 0$$

which can be solved for e_i^{max}:

$$e_i^{max} = \frac{r_{ei}^{max} + r_{ei}^*}{r_i^{max} + b_i}$$

The model system was simulated using the parameter set listed in Table 1. The parameter values were first

estimated from fed-batch and batch cultures. r_i^{max} were estimated from results of metabolic flux analysis on steady-state data. The yield coefficients Y_i are the maximum yields assuming M_1 as pyruvate, M_2 as α-ketoglutarate, and M_3 as ribose 5-phosphate. The half saturation constants (K_i) and the maximum rate of enzyme synthesis were adjusted within the context of the cybernetic model to reflect the dynamics observed experimentally. Although other set of parameters' values gives a good approximation to the observed dynamics, the multiplicity of steady state is very sensitive to the variables' values.

Results and Discussion

Multiplicity of Steady State. The model was used to simulate two continuous cultures initiated from batch and fed-batch modes, respectively. In the fed-batch mode, the concentration of S_1 was set at a constant level of 0.05 g/L. At 100 h the culture was shifted to continuous mode. In the other culture initiated in a batch mode, the concentration of S_1 was initially 0.95 g/L and gradually decreased as it was consumed by the cells. The culture was switched to continuous mode at 75 h. In the continuous mode, the operating conditions were the same for both cultures. The concentrations of S_1 and S_2 in the feed were 0.95 and 0.43 g/L, respectively. The dilution rate was 0.03 h^{-1}.

The two cultures reach two different steady states represented by different concentrations of cells and substrate (Figure 2). The steady-state cell concentration is higher in the culture initiated in a fed-batch mode. A higher level of S_1 is seen in the low cell density steady state, while the levels of S_2 are similarly low at both steady states. The metabolic flux distribution at the two steady states is shown in Figure 3. The specific consumption rates of S_1 are similar at both steady states, while those of S_2 are different. The ratio of M_1 formation from M_2 is very small in both cases. M_1 is formed primarily from S_1.The results thus clearly illustrate that two distinct steady states achieved under identical operating conditions are predicted by a cybernetic model.

Cellular Regulation Leading to Multiple Steady States. The dynamics of the intracellular events leading to different steady states can be better discerned by plotting the time profiles of the intermediate and enzyme concentrations, various reaction rates, and the cybernetic variables (Figures 4 and 5). The initial conditions for enzyme levels and intermediate concentrations for both batch and fed-batch cultures were set to be identical but arbitrary. After the initiation of culture, the two cultures responded rather similarly for a short duration (about 30 h) in terms of M's and e's. This is a result of similar trends in u's and v's initially. Subsequently, although the two cultures both continue to grow at their maximal rate, the substrate profiles became rather different, and u's, v's, M's, and e's all began to diverge. Overall the culture started in a batch mode (low density culture) continues to grow in a relatively abundant environment, especially with respect to the concentration of S_1. This is also clearly demonstrated in the profiles of M's. The levels of M_1, M_2, and M_3 were all higher in the low-density culture throughout the cultivation period. Overall the trends of corresponding cybernetic variable pairs u_i and v_i (such as u_1 and v_1) are similar. Also, r_i and e_i behave similarly. For example, in the batch culture, the enzyme concentrations follow the order $e_1 > e_3 > e_2 > e_4 > e_5$ at steady state although the ratios among them are different; so do the reaction rates with the exception of r_3 and r_4 as response of the changes of v_3^c and v_4^s. Furthermore, at steady

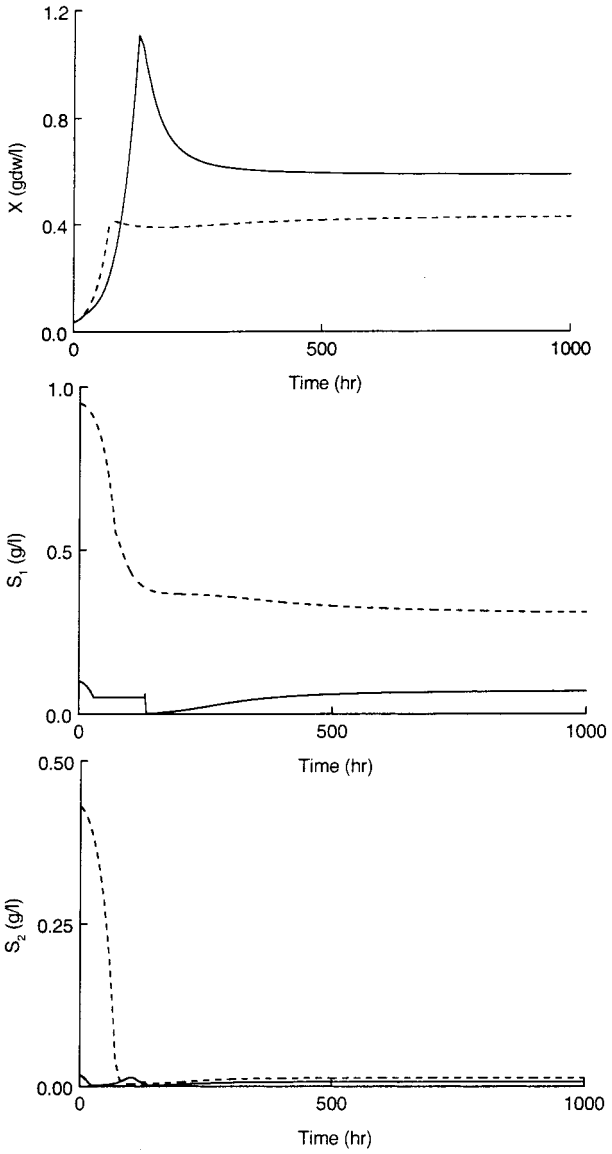

Figure 2. Different steady states in continuous culture initiated as batch (- - -) and fed-batch culture (—).

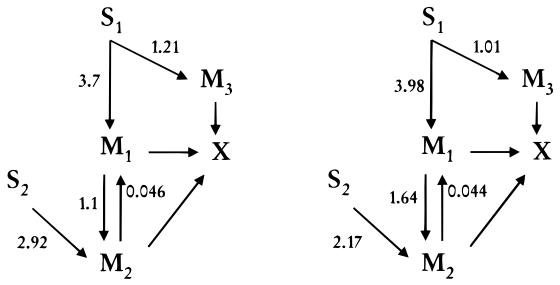

Figure 3. Flux distribution at steady state for cultures started from batch (left diagram) and fed-batch (right diagram).

states both u_5^s and v_5^s are almost zero. The same trend is found between e_i and r_i.

In both cultures, the initiation of continuous operation caused major perturbation. This is reflected in the substrate concentrations, especially visible in S_1 and manifested in all the other variables. In the fed-batch culture, the change in S_1 resulted in a decrease in u_3^c and v_3^c and an increase in u_1^c (v_1^c already at 1.0). The trend was then reversed before all u's and v's settled to steady state. The cybernetic variables u_2^s, u_4^s, u_5^s and v_2^s,

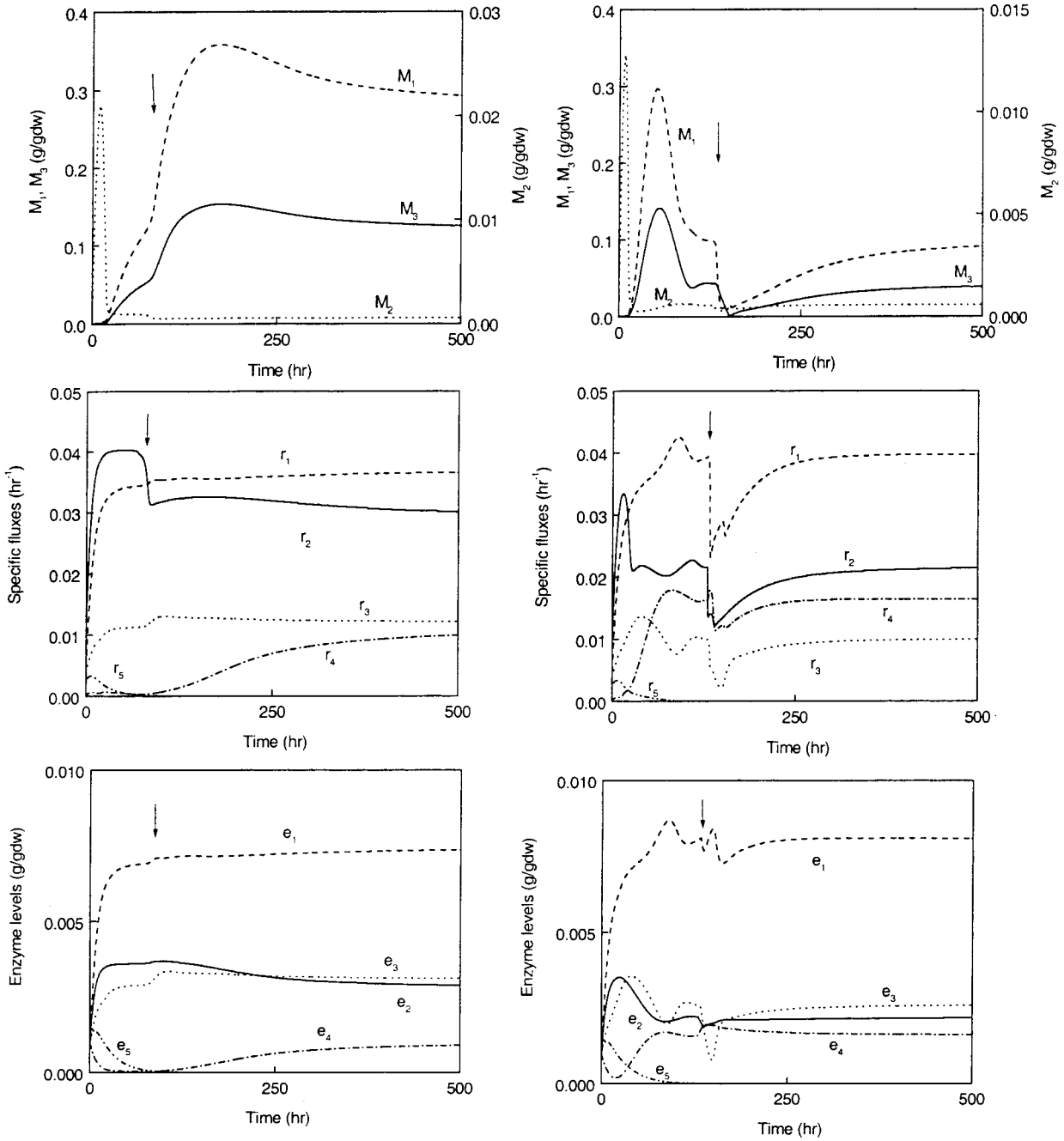

Figure 4. Time profile of enzymes (e_i), intracellular intermediates (M_i), and reaction rates (r_i) in cultures started from batch (left panel) and fed-batch (right panel) modes. The arrows show the start of the continuous mode.

v_4^s, v_5^s did not change in such a wide range as the two complementary cybernetic variables related to S_1 consumption. The values of intermediate (M_i) stayed in the same order of magnitude throughout most of the cultivation, with $M_1 > M_3 > M_2$, except for a short duration in the beginning and another period around 130 h. The change in the order of their magnitude resulted in a more profound response in all cybernetic variables. When the steady state is reached, all variables are constant. In contrast to the fed-batch culture, the cultivation conditions used to simulate the batch culture led to a steady state quickly after switching to a continuous mode.

Influence of the Initial Conditions. The results above demonstrate that the history of the culture affects the resulting steady state. At the time point of switching a continuous mode, the batch culture and fed-batch culture have different sets of S_i, e_i, M_i, and r_i. Those two different sets of initial conditions at the onset of continuous culture led to different steady states. From the results shown in Figure 4 it is clear that during fed-batch culture, although S_i is kept constant, the variables e_i, r_i, and M_i change over time. It is possible that if one switches the culture to a continuous mode at different time points, because of different sets of e_i, r_i, and M_i, the culture may settle in different steady states. To further explore the effect of history, simulations were performed in which a fed-batch culture was switched to a continuous culture at different time points (Figure 6). All cultures reached steady state. Only two steady states were observed. Depending on the timing of switching to a continuous mode or the state of culture at the time of switching, the final steady states are different. However, the cybernetic model presented here is based on a simplified metabolism and does not yet capture all the experimental observations since more that two steady

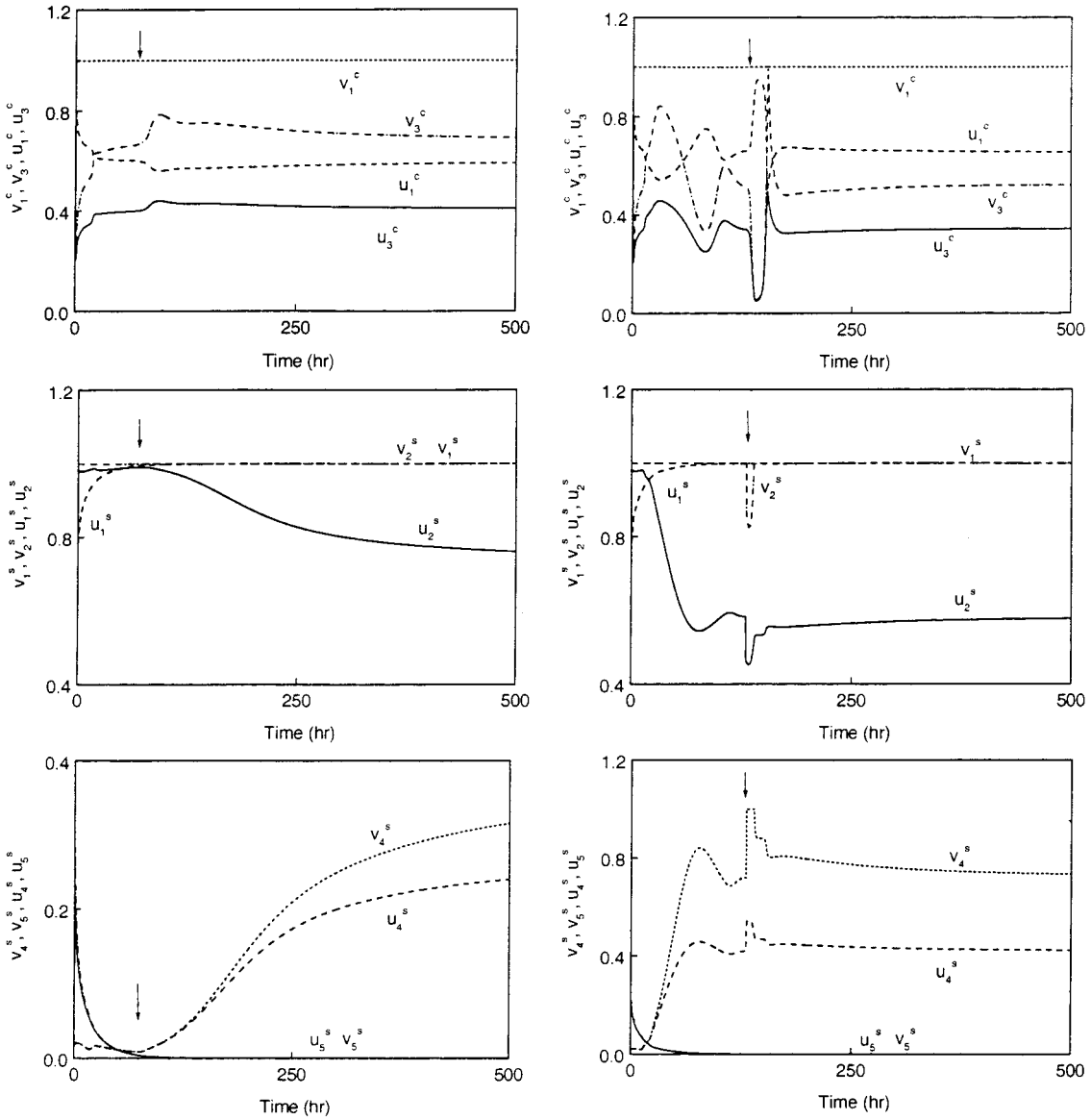

Figure 5. Time profiles of complementary (v_i^c, u_i^c) and substitutable cybernetic variables (v_i^s, u_i^s) in cultures started from batch (left panel) and fed-batch (right panel) mode. The arrows show the start of the continuous mode.

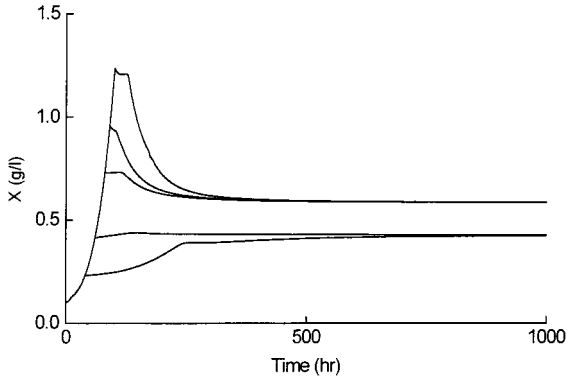

Figure 6. Dependence of the steady state time of switching from fed-batch to continuous culture. Two different steady states are reached.

states have been obtained experimentally from different fed-batch cultures.

The effect of initial conditions on the final steady state was studied further by varying initial enzyme level values in a continuous culture while keeping dilution rate and operating conditions invariant. The level of enzymes e_1 and e_2 are directly affected by a change in the S_1 and S_2 concentration through the cybernetic variables u_1, u_2, v_1, and v_2. The initial levels of these two enzymes were set to vary from e_i^{max} to 10^{-7} g/gdw. Figure 7 shows the dynamics on a two-dimensional concentration phase plot. All the initial conditions tested approach two steady states. Lower values of e_1/e_i^{max} (<0.001) converge to one steady state, whereas the higher values converge to the other, independent of the initial value of e_2/e_2^{max}. The results provide further evidence that, in the cybernetic framework proposed, the initial conditions affect the steady-state reached.

Concluding Remarks

This report has taken a cybernetic approach to attempt to model the multiple steady-state behavior observed in the continuous culture of hybridoma cells. Although the model constructed is a simplified one, it does demonstrate that a cybernetic model offers an illuminating insight into cellular response to the history of the cultivation conditions and achieves different steady states. With more experimental results and a better understanding of metabolic flux distribution, one will be able to further

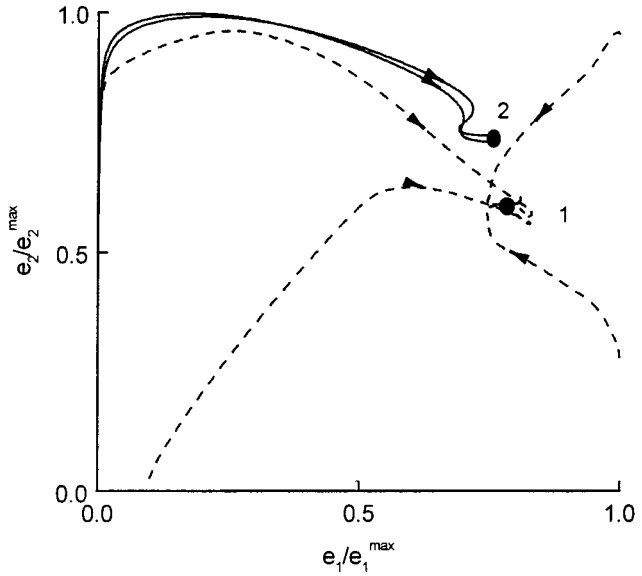

Figure 7. Influence of the initial conditions on the resulting steady states. Values of e_1/e_1^{max} higher than 0.001 converge to steady state 1, whereas lower values converge to steady state 2.

develop the model for process prediction and optimization proposed. The potential utility of a cybernetic model on mammalian cell culture processing is yet to be exploited.

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Notation

b_i degradation constant of the *i* key enzyme (gdw/gdw-h) (gdw denotes gram dry weight)

e_i specific level of the *i* key enzyme (g/gdw)

e_i^{max} maximum specific level of the *i* key enzyme (g/gdw)

K_i saturation constant, as defined in Table 1.

K_{ei} saturation constants for the synthesis of the *i* key enzyme, as defined in Table 1.

K_{g,M_i} growth saturation constant (g/gdw)

M_i specific intracellular level of precursor (g/gdw)

r_i specific flux (g/gdw-h)

r_i^{max} maximum specific flux (g/gdw-h)

r_{ei} specific rate expression of the *i* key enzyme (g/gdw-h)

r_{ei}^{max} maximum specific rate expression of the *i* key enzyme (g/gdw-h)

r_{ei}^{*} constitutive synthesis of the *i* key enzyme (g/gdw-h)

r_g^{max} maximum specific growth rate (gdw/gdw-h)

r_g specific growth rate (gdw/gdw-h)

S_j extracellular growth substrate level (g/L)

u_i cybernetic variable governing the expression of the *i* key enzyme

v_i cybernetic variable governing the activity of the *i* key enzyme

X cell density level (gdw/L)

Y_i yield coefficient, as defined in Table 1.

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