Stoichiometric Medium Design and Nutritional Control in Fed-batch Cultivation of Animal Cells

by

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ABSTRACT

The overall stoichiometric equation governing animal cell growth under optimal culture conditions where by-product formation is minimized was determined with cell composition, energy demand, and eight design parameters, through studies of the metabolic reaction network. The stoichiometric coefficient of nutrient was defined as the minimum requirement for cell growth on a per cell basis. The application of the stoichiometric equation in medium design led to the formulation of stoichiometrically balanced supplemental media for feeding. A feed-on-demand strategy was developed from the stoichiometric equation for nutritional control during the process of fed-batch cultures.

Hybridoma cells, CRL-1606, producing monoclonal antibody were cultivated in fed-batch cultures in T-flasks and in a bioreactor using an integrated medium design approach. Concentrations of glucose, glutamine, and essential amino acids were controlled relatively constant over the culture course through automatic feeding of the supplemental media. As a result of the nutritional control, specific lactate product rate was reduced by 80-fold as compared to a typical batch culture. Less than 5% of the glucose consumption was converted into lactate under the controlled optimal culture condition as compared to 80% in the batch culture. Production of ammonia, and non-essential amino acids was also successfully decreased. Culture viability judged by the maximum viable cell density \((1.7 \times 10^7 \text{ cells/ml})\) and culture life \((550 \text{ hours})\) was significantly increased as compared to the batch and conventional fed-batch cultures. As expected, the product concentration and volumetric productivity were increased from the batch by 50- and 10-fold respectively with the highest monoclonal antibody titer at \(2400 \text{ mg/L}\). The specific product synthesis rate was found to be constant over the fed-batch culture process and unchanged from that of the batch culture.

Material and energy balances were studied with extracelluar measurements from the batch and fed-batch cultures of the hybridoma cells. Hypotheses and
simplifications introduced into the design of the supplemental media were verified. Material balances on essential amino acids showed that their consumption matched well with the demand for protein synthesis under the controlled environment. Isoleucine and leucine could be catabolized for energy production or fatty acid synthesis under an uncontrolled condition. It was found that the uptake of non-essential amino acids from the culture medium reduced ammonia production. Hence addition of non-essential amino acids to the culture medium is essential to optimize the culture performance even though they are not required for cell survival. The TCA cycle was more active, generating more energy under low glucose and glutamine concentrations. About 50% of the total glucose consumption was metabolized in the TCA cycle in a controlled fed-batch as compared to only 4% in the batch culture where 80% of glucose was converted into lactate. This suggests that cell metabolism can be manipulated to maximize culture performance through the stoichiometric medium design and process control.

The stoichiometric approach was also successfully applied to three different Chinese hamster ovary (CHO) cell lines in fed-batch cultures. Significant improvement in the culture performance was achieved in a very short period of time. It is expected that this approach is generally applicable to other systems for quick process optimization.

Thesis Supervisor: Dr. Daniel. I. C. Wang
Title: Institute Professor
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1. Introduction

Mammalian cell cultivation has been one of the most important approaches for the production of various proteins with biological functions for diagnosis and therapy. Animal cell culture has been practiced for more than forty years. Detailed metabolic reactions involved in cell growth are known in biochemistry. Remarkable progress has been obtained in understanding the significance of the primary metabolic reactions through metabolic flux analysis. However, the quantitative nutritional demands for animal cell growth has not been well understood yet. The understanding of cell metabolism in vitro may allow one to manipulate cell growth behavior towards optimal culture performance through process control. This thesis has focused on process design and control by integrating knowledge from biochemistry, chemical engineering, and biotechnology with the goal of improving the mammalian cell culture technology.

1.1 Problems in Traditional Animal Cell Cultures

Animal cell culture has been practiced most often in a batch mode in industry for production of pharmaceutical biologicals. However, many other operating modes such as fed-batch (Glacken, 1987), continuous (Cazzador and Mariani, 1993; DiMasi and Swartz, 1995; Griffiths, 1992; Hiller et al., 1991; Miller et al., 1987; Mohan et al., 1993; Ray et al., 1989), and perfusion (Broise et al., 1991; Chiou et al., 1991; Konstantinov et al., 1996; Mercille et al., 1994; Murakami et al., 1991; Park and Ryu, 1994) have been extensively studied. Simplicity in
process control and operation are some of the obvious advantages for batch culture. On the other hand, the disadvantages such as low product titer and productivity would lead to high costs in downstream purification.

The desired product is usually a protein with biological function secreted by viable cells. Cell growth is a prerequisite for product synthesis. The product concentration is directly proportional to the integrated viable cell concentration with time. Cell growth not only consumes nutrients but also generates by-products, such as ammonia, lactate and non-essential amino acids (NAA). In a batch culture, the theoretical maximum cell density is limited by the amount of nutrients present in the initial medium. The achievable maximum cell density depends on many other parameters. Most often, cells die due to the adverse environment, such as the depletion of key nutrients, accumulation of by-products, or changes of pH and osmolality. Early cell death and low density cause low product concentration in a typical batch culture.

The problem of cell death is often manifested in the basal medium employed in most cell cultures. The first cell culture medium was developed in 1955 by Eagle. Empirical trial and error method was practiced in the later development of culture media. The fact that more than 30 essential nutrients are required for cell survival renders medium optimization very difficult. On the other hand, the depletion of just one essential nutrient could result in cell death. At the end of a batch culture, most of the essential nutrients are often still present in the medium at a significant amount, while some may be completely exhausted. This
indicates that the composition of the basal medium is not optimal to achieve a high cell density, even though cells may grow perfectly well at low densities.

Obviously simply increasing all of the nutrients in the medium without adjusting their ratios is not the solution since this will increase the osmolality to a detrimental level. Furthermore, increased nutrient concentrations could also result in increased by-product formation that leads to low efficiency in nutrient utilization and toxic by-product accumulation. The dilemma between the nutrient depletion and by-product formation cannot be solved in a simple batch culture system.

Fed-batch cultures have been employed to extend the culture span to higher cell densities than typical batch cultures. In most instances, only glucose and glutamine were fed to the cells on the assumption that they were the limiting nutrients (Hu and Himes, 1989; Lindell, 1992). As one can expect, improvement in culture performance was limited due to the depletion of other essential nutrients. In some cases, concentrated complete media without salts were employed for feeding. This strategy can improve the performance but other problems still exist. Because the medium composition is not balanced with respect to the nutritional demands for cell growth, some nutrients accumulate in the medium which leads to an increased osmolality and by-product formation which could cause cell death.

Clearly, fed-batch culture possesses many advantages over batch culture. Nutrient feeding prevents depletion. Dramatic changes of the nutritional
environment can be significantly reduced through balanced feeding. On the other hand, it is no longer necessary to have high initial concentrations of nutrients to achieve a high cell density, since the demands can now be satisfied by the feeding of a supplemental medium. One can further separate the nutritional needs for the rate of growth from the stoichiometric needs for cell replication. This makes it possible to solve the dilemma between by-product formation and nutritional needs to achieve high cell density.

1.2 Thesis Objectives

The goal of this thesis is to design a generic fed-batch culture process to achieve significant improvement in cell culture performance (mainly product concentration) through the studies of metabolic reaction network, stoichiometric nutritional demands, by-product formation, and process control in mammalian cell cultivation. The design of the fed-batch culture process includes three major parts: the initial medium that provides the environment for cell growth, the supplemental medium that satisfies the nutritional demands for cell mass and protein production, and the feeding policy that controls the culture environment over the time course of the cultivation. The culture performance is best judged by the product formation, cell density, nutrient utilization efficiency, and by-product formation. The product concentration and volumetric productivity are two critical parameters which one would like to maximize. This is because the product concentration governs the cost in downstream purification which is an important factor in the overall cost for a purified product.
The concentration and synthesis rate of the product are governed by the living cells. It is therefore essential to achieve and maintain a high viable cell density over an extended period of time to maximize product formation. This mainly depends on the ability to control the cultural environment which is subject to change due to the consumption of nutrients and the accumulation of by-products. The understanding of the metabolic reaction network and the nutritional demands for the syntheses of cellular components, energy, reducing power, and product is therefore vital for an attempt to avoid the depletion of any essential nutrients. In addition, strategies to reduce by-product formation must be integrated into the fed-batch design, otherwise it will be impossible to significantly improve the culture performance. This is because as cells grow, the accumulation of by-products also increases exponentially. At a high cell density, the amount of by-product produced in even a short period of time can be problematic. In this thesis, the formation of by-products is extensively investigated. Strategies are incorporated into the design of the cultural environment, the design of supplemental medium, and the feeding policy.

In addition to the design of a fed-batch system, intracellular metabolism has been explored using extracellular measurements from cell cultures with the aid of the metabolic reaction network through material and energy balances. This study is very useful to obtain a better understanding on the intracellular metabolic events in order to optimize the design of the fed-batch system. It provides the proof to verify assumptions and to justify simplifications introduced into the design of the fed-batch system. It also generates important information for process evaluation.
1.3 Thesis Organization

This thesis is organized into ten chapters. With a brief introduction in Chapter one, extensive literature review is presented in Chapter two. The materials, culture methods, analytic protocols, and calculations are described in Chapter three. In Chapter four, the stoichiometric equation for animal cell growth is elucidated in details. This is followed by the design of culture media and process control in Chapter five using the stoichiometric tools developed in Chapter four. Experimental results obtained in fed-batch cultures with hybridoma cells are then discussed in Chapter six. These data are employed for material and energy balances in Chapter seven. In Chapter eight, the application of the design tools and feeding strategy in CHO cell cultures is discussed. The conclusions are summarized in Chapter nine. In Chapter ten, recommendations for future research are given.
2. Literature Review

2.1 Nutritional Requirements for Animal Cell Growth

Many nutrients are required for mammalian cell growth in vitro. Some of these nutrients provide precursors for the syntheses of cell mass, energy, reducing powder, and product. A balanced salt solution is also essential to maintain an integral cell membrane as well as normal cell functions. In addition, some components contained in animal sera are essential for cell survival even though the required amounts could be very small. Since this thesis is focused on the quantification of the demands for glucose, amino acids, and vitamins for cell growth, other essential nutrients are therefore not reviewed here.

2.1.1 Glucose Metabolism

The metabolism of glucose in mammalian cell cultures has been well studied (Ferrance et al., 1993; Hayter et al., 1993; Konstantinov et al., 1996; Lanks and Li, 1988; Sciandra and Subjeck, 1983; Sugiura, 1992; Wadzinski et al., 1988; Wohlpart et al., 1990; Zielke et al., 1978, 1981). Glucose plays many crucial roles in cell metabolism as shown in Figure 2.1. The glycolytic pathway generates key intermediates for the syntheses of cellular components such as membranes and sugar chains attached to glycoproteins. A significant amount of lactate is produced even under aerobic conditions, especially in the cultivation of transformed cells. The molar ratio of lactate to glucose usually ranges from 1
Figure 2.1 Schematic of simplified glucose metabolism in animal cell cultivation
to 2 when glucose is not intentionally maintained at a low level. The cause for the high glycolytic activity observed in tumor cells is not well elucidated in literature. Graff et al. (1965) suggested that excessive glycolysis is the result of a high influx of glucose due to the absence or ineffectuality of membrane permeability control. Glycolysis was assumed as a defense mechanism against a high intracellular glucose level. This is supported by the facts found with many different cell lines that lactate production was strongly influenced by glucose concentration in culture medium (Hayter et al., 1992; Hu et al., 1987). Cells growing on alternative carbohydrate sources, such as fructose and manose, produce much less lactate (Shi-ping Wang, 1988). On the other hand, Fagan and Racker (1978) found that ADP and P_i regeneration was the rate-limiting factor for the glycolysis in chick embryo fibroblasts.

The oxidative decarboxylation of pyruvate links the glycolysis pathway and the TCA cycle. This irreversible reaction competes with the production of lactate from pyruvate. The regulation of this reaction plays vital roles in the overall glucose and energy metabolism. This topic is out of the scope of this thesis and will not be discussed further.

While glycolysis occurs in the cytosol, the TCA cycle proceeds in the mitochondria only. The TCA cycle provides the majority of the energy demands for primary cells under aerobic conditions, because lactate is generated only during vigorous activities when cells undergo anaerobic metabolism due to oxygen limitation. It is suspected that the TCA cycle is not very active in transformed cells since the majority of glucose is converted into lactate even
when sufficient oxygen is present. The two ATP produced in the conversion of glucose to lactate represents only a very small fraction of the total ATP production from the complete glucose oxidation via the TCA cycle. As a result, glucose utilization is much less efficient in cell culture than in vivo.

The pentose cycle generates various five-carbon sugars and reducing power (NADPH) from glucose-6-phosphate. Among the five-carbon sugars, ribose-5-phosphate is the most important one that plays an essential role in DNA and RNA synthesis. Depending on the demand for cell growth, this pathway can convert glucose into ribose or reducing power.

2.1.2 Glutamine Metabolism

Glutamine is a unique amino acid in cell metabolism and hence will be discussed separately from other amino acids. Its metabolism has been extensively investigated (Katunuma et al., 1967; Lanks, 1986; Levintow et al., 1957; Luan, 1987a; Moreadith and Lehninger, 1984; Patterson et al., 1963; Zielke et al., 1980). A schematic diagram for the glutamine nitrogen metabolism is shown in Figure 2.2. The essential roles of the glutamine amide group in the nucleotide synthesis cannot be replaced by free ammonia or amino groups. However, the amount of glutamine consumed for this purpose is only a small percentage of the total. The glutamine amino group is the major nitrogen donor for the biosynthesis of non-essential amino acids which is catalyzed by aminotransferases. A significant amount of glutamine nitrogen is converted to
Figure 2.2 Schematic of the glutamine nitrogen metabolism in animal cell cultivation
ammonia, a toxic by-product in cell metabolism, via non-enzymatic degradation, glutamine hydrolysis, and glutamate hydrolysis.

The glutamine carbon skeleton shows a different metabolic pathway, called glutaminolysis, from that of the nitrogen groups. A considerable amount of the glutamine carbon enters the TCA cycle as $\alpha$-ketoglutarate. Under a limited glucose condition, glutamine was found to be the major energy source for the growth of human fibroblasts (Zielke et al., 1984). The glutamine carbon skeleton can be found in by-products such as carbon dioxide, alanine, aspartic acid, glutamate, and proline. Because only acetyl-CoA can be completely converted into CO$_2$ in the TCA cycle, carbon chains entering the cycle at any other point have to exit the cycle. The carbon chain of glutamine exits the cycle via the malate shuttle by which malate is converted into pyruvate (McKeehan, 1986).

2.1.3 Other Amino Acids

Thirteen amino acids including glutamine are essential for cell survival in vitro for most cell lines (Eagle, 1955), while only eight (not including glutamine) are indispensable in vivo (Bender, 1985). The seven so called non-essential amino acids can be synthesized mainly from glutamine, the most rapidly consumed amino acid in cell cultures. The amino acid metabolism in animal cell and tissue cultures was investigated extensively in early studies (Chung et al., 1966; Eagle, 1959; Griffiths, 1970; Kruse et al., 1967; Lucy and Rinaldini, 1959; McCarty, 1962; Pasieka et al., 1958a,b; Patterson, 1972; Sinclair and Leslie,
A positive relationship between the consumption of essential amino acids and protein amino acid composition was observed except for valine, isoleucine, and leucine (Kruse et al., 1967). This indicates that the major role of the essential amino acids is to provide precursors for the protein synthesis. The consumption rates of valine, isoleucine, and leucine were affected by their concentrations in the culture medium (Hiller et al., 1994). This can be explained by possible consumption of these amino acids for energy production in the TCA cycle and/or for lipid synthesis. Variations in the production of non-essential amino acids were found among different cell lines (Duval et al., 1991) and under different conditions for the same cell line (Miller et al., 1989b). Alanine is always produced into the culture medium by many tumor cell lines (Duval et al., 1991; Ljunggren and Haggstrom, 1994). Although provisions of NAAs have no significant effects on cell growth rate (Stoner et al., 1972), eliminating NAAs from culture medium results in an increased intracellular level of glutamine that leads to an increased glutamine consumption (Patterson, 1972).

2.1.4 Vitamins

Ten vitamins are essential for cell growth in vitro. Many important enzymes and cofactors are synthesized from vitamins (Stryer, 1988). Nicotinamide is the substrate for the synthesis of NAD, NADH, NADP, and NADPH, while riboflavin provides precursor for the synthesis of FAD and FADH₂. The key coenzyme, CoA, in the pyruvate decarboxylation is synthesized from pantothenate. Pyridoxal phosphate participates in many key metabolic reactions such as the transamination in the NAA synthesis and amino acid decarboxylation. Thiamine
pyrophosphate is a key coenzyme in the pentose phosphate pathway and the
decarboxylation of pyruvate and α-ketoglutarate. Biotin is the CO₂ carrier in
carboxylation reactions. Choline and inositol can be used by cells to synthesize
cell membrane. Choline is also one of the major donors for the one carbon
metabolism. Because of the low requirements and lack of feasible analytic
methods for vitamins, quantification of their demands for cell growth is very
difficult (Lambert and Pirt, 1975). The vitamin composition in culture media was
determined by trial and error (Eagle, 1955). In a continuous hybridoma cell
culture, Hiller et al. (1994) studied the effects of step increases of vitamin B₁ and
B₁₂ on cell growth. Both vitamins showed positive effects on viable cell density
and antibody production.

2.2 Ammonia

2.2.1 Toxicity

It is well known that ammonia is toxic to both animals and plants. The ammonia
level in human blood is usually below 0.05 mM (Bender, 1985), while as high
as 10 mM has been observed in tumor animal cell cultures (Glacken, 1987; Jo
et al., 1990). It appears that the level of ammonia that retards cell growth
depends on the cell line and culture conditions. Doyle and Butler (1990) studied
the effects of culture pH on the toxicity of ammonia in a murine hybridoma
culture and proposed that the toxic species be ammonia instead of ammonium.
With addition of NH₄Cl, Glacken et al. (1986) observed no significant inhibition
at a 4 mM concentration. A 50% reduction in growth rate was observed at 7 mM
of NH₄Cl. More recently, Hassell et al. (1991) studied the effects of ammonia on
several cell lines. At a concentration of 2 mM exogenous ammonia, four cell lines showed little of inhibitory effects; two cell lines showed a 50% growth rate reduction; two other cell lines showed a reduction of greater than 75% in growth rate. In addition, ammonia affects protein glycosylation patterns. Borys et al. (1994) showed that ammonia concentrations in the range of 3 - 9 mM inhibited the N-linked glycosylation in a pH-dependent fashion in a CHO cell culture. Similar inhibitory effects on the O-linked glycosylation were found in a CHO culture by Andersen and Goochee (1995). Cautions should be taken, however, in the interpretation of the above studies where ammonia was spiked at the beginning. In cell culture, the ammonia accumulation occurs over a period of time. Newland et al. (1994) found that gradual addition of ammonia to a batch culture had no inhibitory effect up to 10 mM while the spiking of 2 mM ammonium at the beginning resulted in substantial growth inhibition. This is supported by the results obtained in a CHO culture fed with ammonium chloride where no growth inhibition was observed up to 10 mM ammonia (Hansen and Emborg, 1994). Further evidence was obtained in a hybridoma culture where cells grew normally under 12.5 mM of exogenous ammonia after more than 100 hours of poor growth. These data suggest that some cells may be able to adapt to a high ammonia level.

The mechanism of ammonia toxicity has been studied for many years (Adema, 1989). It was proposed that the toxic effects of ammonia were caused by either a pH change in the lysosome resulted from the uptake of ammonia or in the cytosol by the uptake of ammonium (Adema, 1989). A mathematical model was developed by McQueen and Bailey (1990) to simulate the intracellular pH as a
function of exogenously applied ammonia in an effort to study its inhibitory effects on cell growth. Recently, Ryll et al. (1994) proposed that the intracellular pool of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine was the candidate involved in the mechanism of ammonia inhibition. They found a positive correlation between the pool and the extracellular ammonia level. This mechanism can be employed to explain the following phenomena: the ammonia effects on glycosylation, the pH dependence of ammonia inhibition, and the adaptation of cells to ammonia.

2.2.2 Production

Ammonia is generated mainly from glutamine metabolism. As shown in Figure 2.2, the glutamine amide group can be incorporated into nucleotides or hydrolyzed into ammonia. Two different types of reactions, non-enzymatic degradation and enzymatic degradation by glutaminase, are involved in the hydrolysis of the amide group. The non-enzymatic degradation is a first order reaction with respect to glutamine. The non-enzymatic decomposition rate was found to be a function of pH (Gilbert et al. 1949), temperature (Tritsch and Moore, 1962), and serum concentration (Lin and Agrawal, 1988, Ozturk and Palsson, 1990). The irreversible conversion of glutamine to glutamate is the rate-limiting step in glutaminolysis, the conversion of glutamine to pyruvate (Mckeehan, 1986). The apparent $K_m$ for glutaminase was reported to be 2.2 mM in liver cells (Katunuman et al., 1973), 2 mM in normal rat kidney cells (Goldstein and Schooler, 1967), 4.5 mM in Ehrlich ascites tumor cells (Kovacevic, 1971), and 7.6 mM in highly malignant Ehrlich ascites carcinoma.
(Quesada et al., 1988). Studies with $^{15}$N in a rat kidney cell culture revealed that about 50% ammonia was derived from the glutamine amide group and 30% from the glutamine amino group (Nissim et al., 1987). This suggests that ammonia is produced from the glutamate hydrolysis to yield $\alpha$-ketoglutarate catalyzed by glutamate dehydrogenase. Recently, a study with $^1$H/$^{15}$N nuclear magnetic resonance (NMR) in HeLa and CHO cell cultures showed that ammonia was produced predominantly from the glutamine amide group (Street et al., 1993). This suggests that the glutamate hydrolysis reaction does not proceed towards the side of ammonia production. In a hybridoma cell culture, Zupke and coworkers (1995) reported moderate production or assimilation of ammonia by glutamate dehydrogenase, depending on the culture conditions. Recently, a study on metabolic flux analysis using mass balances in hybridoma cells found that the flux from glutamate to $\alpha$-ketoglutarate was almost zero or even slightly reversed (Bonarius et al., 1996). This is consistent with the results presented by Street et al. (1993) and Zupke et al. (1995), but contrary to what was found by Nissim et al. (1987).

### 2.2.3 Methods to Reduce Ammonia Toxicity

Two major types of strategies have been studied to avoid the ammonia toxicity. The first one is to reduce its production through the manipulation of the culture conditions. The other is to remove ammonia from the culture vessel. A strategy of reducing ammonia production was first proposed by Glacken et al. (1986) and applied in a glutamine limited fed-batch culture system. This strategy was further explored by Ljunggren and Haggstrom (1990, 1992, and 1994) with a
series studies using glutamine limited fed-batch cultures. Significant reduction in ammonia formation was achieved while improvement in the culture performance was limited. The effects of the glutamine concentration on the ammonia production in hybridoma batch cultures were studied by Dalili et al. (1990). A membrane-based approach was developed by Brose and Ekeren (1990) to remove ammonia based on diffusion. Another tactic uses electric means to remove charged metabolites including ammonium and lactate through filter paper and an agarose-filled salt bridge (Chang et al., 1995a, b). Both approaches suffer from the same scale-up problem. Removal of ammonium from the culture medium for mouse embryo development was developed using in situ enzymatic conversion to glutamate (Lane and Gardner, 1995).

2.3 Lactate

2.3.1 Toxicity

The toxic mechanism of lactate on cell growth is not fully understood. However, it is widely agreed that the potential of lactate to change extracellular pH and osmolality is responsible for its toxic effects. The conversion of one mole of glucose to two moles of lactate is coupled with the generation of two moles of protons. These have to be neutralized by base addition otherwise a pH change will result. In addition to the one extra mole of lactate produced per mole of glucose consumed, the base addition also contributes to the osmolality increase. The effects of lactate addition to culture medium on the initial hybridoma cell growth rate were extensively studied by Glacken and coworkers
(1988). Through statistical design, the effects of lactate and other factors on cell growth were incorporated into the expression of cell growth rate. Recently, Chang (1994) found that addition of 40 mM sodium lactate had no considerable effect on cell growth rate or the maximum cell density, while 80 mM caused significant reduction in both cell growth rate and the maximum cell density. The inhibitory effects of lactate and ammonia on cell growth were found interactive by Hassell and coworkers (1991) when lactate concentration exceeded a certain level. This is probably because lactate accumulation may destroy the pH buffer capacity of the culture medium, which could enhance the sensitivity of the intracellular pH to ammonia. With proper pH control, osmolality increase was found to be responsible for the inhibitory effects of lactate in a lactate-controlled fed-batch culture (Omasa et al., 1992). Evidence from many different cases indicates that lactate itself has little negative influence on cell growth (Chang, 1994; Kurano et al., 1990a; Omasa et al., 1992).

2.3.2 Production

Lactate is generated mainly from glucose. Experiment with radiolabeled compounds revealed that over 90% of glucose was transformed into lactate in an ascites-tumor cell culture (Lazo, 1981). As discussed in the glucose metabolism, lactate production from glucose is affected strongly by glucose concentration (Miller et al., 1989a). Interestingly, the ratio of lactate-to-glucose increases with cell growth rate as shown by Luan et al. (1987b) in a hybridoma culture. Since the glutamine carbon skeleton entering into the TCA cycle must exit via the malate to pyruvate pathways, glutamine can also contribute to the
lactate formation (McKeehan, 1986). This is in agreement with the observation that glutamine-limited fed-batch culture (with normal glucose) generates less lactate (Ljunggren and Haggstrom, 1992).

2.3.3 Methods to Reduce Lactate Toxicity

There are two approaches to reduce lactate toxicity. Various attempts have been made to remove lactate from culture vessels. These include dialysis (Kasehagen et al., 1991; Kurosawa et al., 1991; Lindardos et al., 1992) and electric means (Chang et al., 1995a). The second approach involves the manipulation of culture environment for lactate reduction. Glucose-limited fed-batch culture is the most effective approach found in the literature (Glacken et al., 1986; Hu and Himes, 1989; Hu et al., 1987; Kurokawa et al., 1994; Ljunggren and Haggstrom, 1994). Using an on-line estimation of cell concentration, Glacken et al. (1986) developed an automatic feeding strategy for glucose control in a fed-batch hybridoma culture. The effects of low glucose and glutamine concentrations on the lactate and ammonia formation were studied by Ljunggren and Haggstrom (1994) using constant feeding flow rates of glucose and glutamine in a fed-batch hybridoma culture. Low glucose concentration not only reduces the glycolytic flux that generates pyruvate, the substrate for lactate formation, but also reduces the activity of the intracellular lactate dehydrogenase, the enzyme catalyzing the conversion of pyruvate to lactate (Kurokawa et al., 1994).
2.4 Medium Development

One of the most important considerations in animal cell cultivation is in determining the composition of the culture medium. Unlike micro-organisms, animal cells are very sensitive to their culture environment. The concentrations of glucose, amino acids, and vitamins in culture media not only affect cell growth rate, but could also stoichiometrically limit the maximum cell density obtainable in a batch culture.

The sophisticated nutritional requirements of animal cells impose real challenges on the design of a proper medium needed to achieve optimal cell growth and to attain a high cell density. Animal cell metabolism in vitro is not only complicated, but also variable, depending upon the culture conditions (nutritional and operational) as well as characteristic differences in cell lines. This renders the medium design more difficult. At the early stage of medium development, the goal was to determine all of the nutrients essential for in vitro cell survival (Eagle, 1955; Morgan et al., 1950). Ever since Eagle (1955) developed a minimal essential medium (MEM), many efforts have been devoted to kinetic studies for medium optimization (Batt and Kompala, 1989; Bree et al., 1988; Glacken et al., 1988; Miller et al., 1988). Most commercial culture media were designed for the purpose of achieving a maximum cell growth rate (Lambert and Pirt, 1975).

A simple attempt to avoid nutrient depletion is to increase the nutrient concentrations in the medium without changing the medium osmolality by adjusting the salt concentrations accordingly. Significant improvement in cell
density and product titer was attained in a hybridoma culture using a fortified RPMI 1640 medium (Jo et al., 1990). This is not a generic approach, however, since high glucose and glutamine concentrations lead to high lactate and ammonia production as shown by Chang et al. (1995b). The effects of cell growth on the change of the culture environment which were often neglected should be considered in the medium design.

Recently, glutamine-based dipeptides (alanyl-glutamine and glycyl-glutamine) have been employed to replace free glutamine in culture media (Butler and Christie, 1994; Christie, 1994; Jayme, 1991). These dipeptides can be hydrolyzed by cytosolic peptidases releasing free glutamine for cell growth. The advantage of this approach is that the dipeptides are resistant to non-enzymatic deamination even at high temperature, and hence are less ammoniagenic. This allows long-term storage and autoclaving as a means for medium sterilization. Because free glutamine concentration increases rapidly in the culture medium (Christie, 1994), non-enzymatic glutamine deamination still occurs during the culture process. Hence, reduction in ammonia production from glutamine is limited. This approach also has the disadvantage that a significant amount of alanine or glycine is remained in the culture medium due to the much less requirement for cell growth as compared to glutamine. In high cell density culture where the total glutamine consumption can be as high as 75 mM (see Chapter 6), the amount of alanine or glycine generated from dipeptides will elevate the osmolality to a detrimental level. Other approaches to reduce ammonia production involve complete elimination of glutamine from culture media via an adaptation procedure (Kurano et al., 1990a,b) or
transfection of a glutamine synthetase marker (Bebbington et al., 1992). Almost no ammonia is produced when glutamine-free medium is employed (Birch, 1994). However, it should be noted that not every cell line can grow in glutamine-free media (Hassell, 1990).

The stoichiometry governing microbial growth is often simpler than that of animal cell growth and has undergone detailed studies (Cooney et al., 1977). However, the stoichiometric nutritional demands for animal cell growth have not yet been extensively studied. Thus, the nutrient concentrations in the conventional media were primarily determined experimentally through trial and error. The understanding of the cellular needs for nutrients will allow one to quantitatively design culture media in such a way that depletion or accumulation of nutrients may be avoided. This is an important step toward an optimized culture process.

It should be mentioned that serum-free media have been investigated for many years (Barnes and Sato, 1980; Butler, 1986; Darfler and Insel, 1983; Glassey et al., 1988; Kawamoto et al., 1983; Kitano, 1991; McKeenan et al., 1977; Schneider, 1989; Yssel et al., 1984). Due to concerns with downstream purification and possible virus contamination, serum-free or even animal-derived-product-free media are desirable for the manufacturing of therapeutic biologicals using animal cell culture.
2.5 Fed-batch Culture

Fed-batch culture has been studied by many investigators to increase cell density, culture span, and productivity (Bushell et al., 1994; Glacken et al., 1986; Hansen et al., 1993; Luan et al., 1987c). The addition of essential nutrients usually results in extended cell growth which leads to increased product formation. The effects of various combinations of glutamine, essential amino acids, vitamins, and serum addition were studied by Luan and coworkers (1987c). Due to the omission of glucose in the supplemental medium, improvement in culture performance was only marginal. Nevertheless, this early attempt stimulated numerous studies on the design of supplemental media for fed-batch cultures. Realizing the important roles of glucose and glutamine in energy metabolism and by-product formation, Glacken and coworkers (1989) employed an empirical approach to determine the glucose and glutamine concentrations in an otherwise 4X concentrated complete medium (DMEM without salts except sodium chloride) for feeding to prevent glucose or glutamine depletion. Mathematical model was employed to determine a feeding policy.

2.5.1 Supplemental Medium

Various supplemental media have been developed in attempts to extend culture span for high culture productivity (Chang, 1994; Glacken, 1987; Zhou et al., 1995). Using a 10X concentrated basal IMDM without salts for feeding in a culture of NS0 cells transfected with a glutamine synthetase vector system, Bibila and coworkers (1994) were able to obtain a 7-fold increase in product
titer. The unique glutamine synthetase vector eliminated ammonia production and the requirement for glutamine. Because the nutrient ratios in the basal medium were not balanced with the demands for cell growth, accumulation of some nutrients and by-products was observed which resulted in a significant increase in osmolality. This approach was improved through iterative analysis of spent culture medium to adjust the nutrient ratios in the supplemental medium (Bibila and Robinson, 1995). Although, the concentrated supplemental medium and feeding were empirically designed, substantial improvement were achieved in the final antibody titer. Similarly, Jo et al. (1993) employed a fortified RPMI medium with a reduced concentration of sodium chloride as a supplemental medium. Significant improvement in the product titer was also achieved with an empirical feeding policy. Recently, Chang and coworkers (1994b) improved the nutrient enrichment approach by using electrical means to remove ammonia and lactate.

In an attempt to balance the nutritional demands for cell growth, experimentally measured nutrient consumption rates from batch cultures were employed to determine the ratios of key nutrients (glucose, and some amino acids) in the supplemental medium (Fike et al., 1993). This provides an effective, rapid, and simple approach to solve the nutrient depletion problem. The disadvantages of this approach are very obvious. Nutrient consumption rate is affected by culture condition, and hence may not be applicable to different circumstances. More importantly, batch culture is not an efficient process due to the enormous production of by-products.
2.5.2 Feeding Policy

The development of a feeding strategy is very critical to optimize the culture performance. Various strategies have been developed in the past to achieve different purposes. Mathematical models were employed for programmed feeding to ensure a low level of glucose or glutamine for by-product reduction (Hu et al., 1987; Glacken et al., 1988). Ljunggren and Haggstrom (1994) used constant feeding rates of glucose and glutamine to obtain a glucose and glutamine limited culture environment. The nutritional requirements increase exponentially with cell growth. Constant feeding rate cannot meet the nutritional demands for cell growth. An automatic feeding strategy was devised by Kurokawa and coworkers using an on-line HPLC system for nutrient monitoring. In a similar fashion, Zhou and Hu (1994) developed an automatic feeding strategy using on-line measured dynamic oxygen uptake rate. It should be noted that only one parameter can be controlled through the feeding rate if the supplemental medium is not properly formulated. Usually, glucose concentration is chosen to be the key parameter for process control due to its central roles in cell metabolism.

2.6 Monitoring and Control

The control of animal cell culture systems is very important to maximize product formation and to ensure reproducibility as required by Good Manufacturing Practice (GMP). On-line monitoring provides essential information to allow automatic control of culture parameters such as pH, DO, and nutrient
concentrations. Various monitoring approaches have been developed in animal cell culture systems (Jenkins, 1995; Konstantinov et al., 1992; Lee et al., 1991; Renneberg et al., 1991; Ruaan et al., 1993) and were reviewed by Junker and coworkers (1994). A multi-channel flow injection analysis system was developed by Pol and coworkers (1994) and Blankenstein et al. (1994) for the on-line monitoring of glucose, glutamine, glutamate, lactate, and ammonia in animal cell cultures. The accurate measurements of key nutrients and by-products facilitate the control of these key parameters over the course of a cell culture. Cattaneo and Luong (1993) developed a chemiluminescence fiber optic biosensor for on-line monitoring of glutamine. A dynamic method for oxygen uptake rate (OUR) measurement was developed by Lindell (1992) to correlate with viable cell density for nutritional control in a bioreactor. Real-time monitoring of OUR was developed by Yoon and Konstantinov (1994) using two dissolved oxygen probes in animal cell culture bioreactors. Dorresteijn and coworkers (1996) developed a software sensor to monitor the volumetric biomass activity using an estimated ATP production rate from OUR and lactate production. With on-line measurements of optical density, OUR, and base addition for the estimation of lactate production, Zhou and Hu (1994) were able to characterize the physiological states of hybridoma cells. In addition, on-line monitoring of the product concentration in a perfusion culture was practiced by Polzius et al. (1993) using a flow injection analysis system.
2.7 Metabolic Reaction Network

Metabolic flux analysis has been applied to microbial systems for the analysis of flux distributions (Cortassa et al., 1995; Goel et al., 1993; Marx et al., 1996; Pons et al., 1996; Vallino and Stephanopoulos, 1993) and for the optimization of product formation (Stephanopoulos and Vallino, 1991; Varma et al., 1993). Recently, Zupke and Stephanopoulos (1995) presented an intracellular flux analysis using kinetic material balances and confirmed fluxes with carbon-13 NMR spectroscopy measurements in a hybridoma culture system. Using carbon-13 NMR spectroscopy in a hollow fiber bioreactor, Mancuso et al. (1994) and Sharfstein et al. (1994) studied the primary metabolic pathways in hybridomas. A reaction network for intermediates in hybridomas was employed by Savinell and Palsson (1992) to identify the limiting nutrient in a cell culture process. With measured uptake and production rates of nutrients, product, and by-products from a continuous culture, Bonarius and coworkers (1996) carried out a metabolic flux analysis of hybridoma cells with the assistance of mass balances. The use of flux analysis could be very useful for identifying the key metabolic reactions provided that a sufficient number of accurate measurements are available. One of the disadvantages of the metabolic flux analysis is that it requires a set of flux measurements, such as specific nutrient consumption rates and specific production rates of biomass and products. However, these measurements are functions of growth rate and hence are not constant during a culture process. Furthermore, little progress has been made to optimize culture performance using the knowledge obtained from metabolic flux analysis.
2.8 Material and Energy Balances

Material balances have been routinely practiced in microbial systems for many years (Wang, 1977, Cooney et al., 1977, Shuler and Kargi, 1992). Usually, elemental balance equations on carbon, nitrogen, oxygen, etc. are sufficient to determine the material balances for these fermentations. The stoichiometry for microbial cell growth and product formation was examined using material balances by Cooney and coworkers (1977) to predict fermentation parameters for automatic process control. Heijnen and Roels (1979) applied elementary balancing methods in combination with kinetic equations to formulate an unstructured model for the production of penicillin in a fed-batch process. The pioneering work of Bauchop and Elsdon (1960) has inspired a tremendous number of studies on the ATP requirement for the formation of microbial cellular components (Papoutsakas, 1984; Roels, 1980; Shuler and Kargi, 1992; Stouthamer, 1973, 1977, 1979a,b). Many different approaches have been taken to estimate the theoretical ATP requirement in microbial fermentation systems. Based on microbial cell compositions, Stouthamer (1977) studied the theoretical ATP yields for different microorganisms. The effects of different carbon and energy sources on the theoretical ATP yields were also investigated. Roels (1980) developed a simple material and energy balance model to study the microbial growth on substrates with different degrees of reduction. A stoichiometric equation was derived by Papoutsakis (1984) for butyric acid bacterium fermentation. This stoichiometric approach was further
studied and employed in other fermentation by Papoutsakis and Meyer (1985a,b).

In contrast, detailed material balances in animal cell culture have not been actively studied, mainly due to the complexities of animal cell metabolism and the nutritional requirements. The general elemental balance approach practiced in microbial system is insufficient to define an animal cell culture system. Animal cell metabolism is complicated and is not fully understood. These complexities render the material balances for animal cell cultivation a difficult problem to solve. Nevertheless, detailed material balance analysis in animal cell culture system would be desirable to provide insights towards a better understanding of animal cell metabolism.

Incomplete material balances have also been incorporated into kinetic models (Batt and Kompala, 1989; Glacken et al., 1989; Frame and Hu, 1991a,b; Tremblay et al., 1992; Zeng, 1995; Zeng and Deckwer, 1995; Zhou et al., 1995). Batt and Kompala (1989) developed a structured kinetic model for hybridoma cells. Material balances were employed to estimate the intracellular pools of lumped amino acids, nucleotides, lipids, and proteins. The growth kinetics was then expressed as a function of the production rates of these intracellular pools. In other cases, constant specific nutrient consumption rates or nutrient yields were assumed for the estimation of nutrient concentrations that were used to express the cell growth rate (Glacken et al., 1989; Frame and Hu, 1991a,b; Tremblay et al., 1992). Realizing that both specific nutrient consumption rates and nutrient yields are not constant and are affected by nutrient concentrations,
Zeng (1995) developed a model to correlate nutrient consumption and product formation with nutrient concentrations. In general, when incomplete material balances were employed in these kinetic studies, empirical methods had to be used and only a few nutrients and products were considered.

Without detailed material balances, an estimation of ATP production relied mainly on the measured lactate production rate and oxygen uptake rate (Glacken et al., 1986; Miller et al., 1989a,b; Ozturk and Palsson, 1991a,b). However, the cell density and growth rate of animal cells are often lower by orders of magnitude than most microorganisms. This results in a very low OUR, and renders the OUR measurement very difficult and inaccurate in animal cell culture systems. Due to the above difficulties, energy metabolism has not been studied extensively or in details in animal cell culture systems.
3. Materials and Methods

3.1 Cell Lines and Growth Conditions

3.1.1 Hybridoma Cell Stock Preparation

A mouse-mouse hybridoma cell line, CRL-1606, producing anti-fibronectin IgG monoclonal antibody, was obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were thawed and diluted ten-fold with Iscove's Modified Dulbecco's Medium (IMDM) (Sigma Chemical, St. Louis, MO) supplemented with 5% dialyzed fetal bovine serum (JRH Biosciences, CA), 4 mM glutamine (Sigma), and antibiotics (10 units/ml penicillin, and 10 µg/ml streptomycin, Sigma). Cells were centrifuged and resuspended in 10 ml fresh medium to remove dimethy sulfoxide (DMSO). Cells were cultivated in T-175 flasks in a 37 °C incubator with a 10% CO₂ overlay, and were then diluted 7-fold every 2 days with fresh IMDM, and scaled up to 200 ml spinner flasks. When the viability reached 98%, the cells were centrifuged at 700-1000 rpm with an IEC Centra-4B centrifuge (International Equipment Company) for 5-10 minutes and resuspended with IMDM supplemented with 10% serum and 5% DMSO to a density of 5×10⁶ cells/ml. Vials containing 1 ml of the cell suspension were frozen at -20 °C for one hour and subsequently placed in -70 °C for 12 hours prior to storage in a liquid nitrogen cell bank as stock cultures. For each experiment, one vial was taken from the cell bank and was cultured in the same fashion as in the preparation of cell stock until the viability reached
98% when the experiment was initiated. Cells were centrifuged at 700-1000 RPM for 5-10 minutes prior to inoculum.

3.1.2 CHO Cell Stock Preparation

An anchorage-dependent CHO cell line producing human interferon-γ was previously adapted to suspension in RPMI containing 0.75% FBS in the Biotechnology Process Engineering Center at MIT. One frozen vial was thawed and diluted ten-fold in a 20 ml shake flask with JRH protein free medium supplemented with 4 mg/L bovine pancreas insulin (Sigma cat. # I1882), 2 mg/L human holo-transferrin (Sigma cat. # T2158), and 2 g/L Primatone RL (Quest International, NY). The shake flask was placed in a shaker with an agitation rate of 70 rpm and maintained in a 37 °C incubator with a 10% CO₂ overlay. Cells were diluted 4-fold after reaching over 1.2×10⁶ cells/ml. The same dilution was carried out every 2-3 days in 100 ml shake flasks and then into 200 ml spinner flasks until a viability of 95% was attained. For stock preparation, cells were centrifuged at 700-1000 rpm with an IEC Centra-4B centrifuge for 5-10 minutes and resuspended with fresh JRH medium with 5% DMSO to a density of 2×10⁷ cells/ml. It was then diluted 2 fold with conditioned protein free medium obtained from the culture where the cells were initially growing. Vials containing 1 ml of the cell suspension were frozen at -20 °C in a cell freezing device that was maintained at -70 °C for 24 hours prior to storage in a liquid nitrogen cell bank as stock cultures. A new vial was taken from the cell bank for each set of experiment. The cells with a viability of 95% were obtained the same way as in
the preparation of cell stock. These cells were then used for inoculum after centrifuged at 700-1000 RPM for 5-10 minutes.

3.2 Preparation of Culture Media

Three similar initial media were developed specifically for fed-batch hybridoma cultures. The nutrients contained in these media were not sufficient to maintain cell growth for a long time without the addition of supplemental nutrients. The cell growth kinetics and the reduction of undesired by-products were considered when these media were designed. The composition and other parameters are listed in Table 3.1. In the preparation of these media, chemicals purchased from Sigma were dissolved in the following order: amino acids, glucose, and then inorganic salts except CaCl₂ and MgSO₄. Vitamins were prepared as a 1000-fold concentrated solution. The pH of the vitamin stock solution was adjusted to 10-11 with 1N NaOH solution. Because of the low solubility for some of the salts and precipitating reactions among Ca²⁺, Mg²⁺, CO₃²⁻, and PO₄³⁻, CaCl₂ and MgSO₄ were then added slowly while stirring as the last step. Acid or base solution was employed to adjust the pH to 7.2. Osmolality was measured to ensure that it was in the range of 260 - 290 mOsm/kg.

The initial medium developed for CHO cells was serum-free and at a low protein concentration. The composition is shown in Table 3.2. Twenty-three stocks solutions were prepared according to Tables 3.3 and 3.4. Due to the low solubility, the pH in stock III was raised to 10-11 with 1N NaOH solution. Ferric
Table 3.1 Composition of initial media for hybridoma cultures

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<td></td>
<td></td>
</tr>
<tr>
<td>tryptophan</td>
<td>245.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tyrosine</td>
<td>417.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>valine</td>
<td>281.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Stock IV</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CuSO₄.5H₂O</td>
<td>0.7491</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂SeO₃</td>
<td>17.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnSO₄.7H₂O</td>
<td>201.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Stock V</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NH₄VO₃</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂MoO₄</td>
<td>0.00035</td>
</tr>
</tbody>
</table>
Table 3.4 Concentrations of individual stock solutions for Init-D

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentrations</th>
<th>Fold of 1X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic Acid</td>
<td>0.25 g/L</td>
<td>3333</td>
</tr>
<tr>
<td>Lipoic Acid</td>
<td>8 mg/L</td>
<td>40</td>
</tr>
<tr>
<td>Putrescine</td>
<td>80 mg/L</td>
<td>500</td>
</tr>
<tr>
<td>Pluronic F-68</td>
<td>100 g/L</td>
<td>100</td>
</tr>
<tr>
<td>Ferric Citrate</td>
<td>61g/L</td>
<td>500</td>
</tr>
<tr>
<td>EDTA</td>
<td>20 mM</td>
<td>1000</td>
</tr>
<tr>
<td>Ethanolamine.3HCl</td>
<td>3g/L</td>
<td>1000</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>20 mg/L</td>
<td>200</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>500 mg/L</td>
<td>1000</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>5 g/L</td>
<td>10000</td>
</tr>
<tr>
<td>Tween 80</td>
<td>2 g/L</td>
<td>10000</td>
</tr>
<tr>
<td>Tocopherol acetate</td>
<td>2 g/L</td>
<td>10000</td>
</tr>
<tr>
<td>LiCl</td>
<td>100 mg/L</td>
<td>5000</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>0.2 mg/L</td>
<td>2000</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>200 mM</td>
<td>200</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>120 mM</td>
<td>200</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>106 mg/L</td>
<td>20</td>
</tr>
<tr>
<td>Insulin</td>
<td>2 g/L</td>
<td>1000</td>
</tr>
</tbody>
</table>
citrate was dissolved in water after heating at 100 °C for half an hour. Difficulty in dissolving some of the nutrients was also encountered while preparing the stock solution listed in Table 3.4. Again, 1N NaOH solution was added slowly to ensure total dissolution of the solutes. Due to possible degradation when stored as a solution, powdered Primatone RL was used when the initial medium was prepared. The medium pH and osmolality were adjusted in the same fashion as stated above.

Three generations of supplemental media (Table 3.5) were designed according to the stoichiometric equation described in Chapter four for hybridoma fed-batch cultures. Stock solution for aspartic acid and tyrosine was prepared in a 10 fold concentration at pH 11. Similarly, riboflavin and folic acid stock solution was prepared in a 100-fold concentration at pH 11. The other eight vitamins were dissolved together in 100-fold concentrations at pH 9. In the preparation of these media, amino acids and glucose were dissolved first, followed by the addition of the concentrated stock solutions, and then the other nutrients. The final pH was adjusted to 9.0 to avoid precipitation during storage in a refrigerator. Freezing of the supplemental media should be avoided since precipitation will result. One supplemental medium, (Sup-IV, see Table 3.5) was also developed for CHO culture using cell composition obtained from hybridoma cells. Stock solutions were prepared according to the formulas presented in Table 3.6. Glutamine and asparagine powders were added last in the preparation of Sup-IV. This is because both glutamine and asparagine are unstable in solution. The final pH was also increased to 9 to ensure stability.
<table>
<thead>
<tr>
<th>Components</th>
<th>Sup-I mM</th>
<th>Sup-II mM</th>
<th>Sup-III mM</th>
<th>Sup-IV g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>7.7</td>
<td>0.339</td>
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<td></td>
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<tr>
<td>CuSO₄·3H₂O</td>
<td>0.0015</td>
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<td></td>
</tr>
<tr>
<td>Na₃SeO₃</td>
<td>0.035</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.403</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric Citrate</td>
<td>0.244</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄</td>
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<td>0.056</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td>240</td>
<td>115.4</td>
<td>289</td>
<td>48.24</td>
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<tr>
<td>alanine</td>
<td>11.63</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>arginine</td>
<td>6.07</td>
<td>7.4</td>
<td>14.9</td>
<td>2.99</td>
</tr>
<tr>
<td>asparagine·H₂O</td>
<td>5.68</td>
<td>3.08</td>
<td>11.7</td>
<td>1.29</td>
</tr>
<tr>
<td>aspartate</td>
<td>18.62</td>
<td>8.64</td>
<td>14.8</td>
<td>1.74</td>
</tr>
<tr>
<td>cysteine</td>
<td>3.62</td>
<td>3.49</td>
<td>7.0</td>
<td>0.98</td>
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<tr>
<td>glutamate</td>
<td>0</td>
<td>5.01</td>
<td>0</td>
<td>0.59</td>
</tr>
<tr>
<td>glutamine</td>
<td>16.55</td>
<td>41.9</td>
<td>111.7</td>
<td>20.26</td>
</tr>
<tr>
<td>glycine</td>
<td>14.3</td>
<td>5.41</td>
<td>5.9</td>
<td>0.97</td>
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<tr>
<td>histidine·HCl·H₂O</td>
<td>2.71</td>
<td>2.69</td>
<td>5.4</td>
<td>1.31</td>
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<tr>
<td>isoleucine</td>
<td>5.94</td>
<td>5.27</td>
<td>10.6</td>
<td>1.60</td>
</tr>
<tr>
<td>leucine</td>
<td>9.69</td>
<td>10.1</td>
<td>22.9</td>
<td>3.08</td>
</tr>
<tr>
<td>lysine·HCl</td>
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<td>8.47</td>
<td>17.1</td>
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<tr>
<td>methionine</td>
<td>2.2</td>
<td>2.71</td>
<td>5.5</td>
<td>0.94</td>
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<tr>
<td>phenylalanine</td>
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<td>3.98</td>
<td>8.0</td>
<td>1.53</td>
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<td>5.94</td>
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<td>5.0</td>
<td>0.58</td>
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<td>serine</td>
<td>9.17</td>
<td>3.19</td>
<td>11.5</td>
<td>0.36</td>
</tr>
<tr>
<td>threonine</td>
<td>7.76</td>
<td>7.15</td>
<td>14.4</td>
<td>1.98</td>
</tr>
<tr>
<td>tryptophan</td>
<td>1.42</td>
<td>1.37</td>
<td>4.0</td>
<td>0.65</td>
</tr>
<tr>
<td>tyrosine·2Na·2H₂O</td>
<td>4.52</td>
<td>3.23</td>
<td>6.5</td>
<td>1.96</td>
</tr>
<tr>
<td>valine</td>
<td>8.91</td>
<td>7.51</td>
<td>15.1</td>
<td>2.04</td>
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<tr>
<td>D-biotin</td>
<td>0.069</td>
<td>0.05</td>
<td>0.10</td>
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<tr>
<td>choline chloride</td>
<td>3.402</td>
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<td>4.77</td>
<td>0.54</td>
</tr>
<tr>
<td>folic acid</td>
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<td>0.093</td>
<td>0.17</td>
<td>0.063</td>
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<tr>
<td>myo-inositol</td>
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<td>1.35</td>
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<td>0.37</td>
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<td>niacinamide</td>
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<td>0.069</td>
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<td>0.95</td>
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<td>0.033</td>
<td>0.06</td>
<td>0.019</td>
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<tr>
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<tr>
<td>D-Ca pantothenate</td>
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<td>0.11</td>
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<td>vitamin B12</td>
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<td>0.007</td>
<td>0.012</td>
<td>0.01</td>
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<tr>
<td>pluronic F-68</td>
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<td>1.0</td>
</tr>
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<td>hypoxanthine</td>
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<td>0.0005</td>
</tr>
<tr>
<td>lipoic Acid</td>
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<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>ethanolamine·3HCl</td>
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<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>glutathione (reduced)</td>
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<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>putrescine</td>
<td></td>
<td></td>
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<td>0.0016</td>
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</table>
Table 3.6 Formulas of stock solutions for supplemental medium Sup-IV.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration mg/L</th>
<th>Components</th>
<th>Concentration mg/L</th>
</tr>
</thead>
<tbody>
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<td><strong>Stock S-I</strong></td>
<td>4X</td>
<td><strong>Stock S-III</strong></td>
<td>100X</td>
</tr>
<tr>
<td>Alanine</td>
<td>0</td>
<td>d-Biotin</td>
<td>1.93</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.9873</td>
<td>Choline Chloride</td>
<td>54.242</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.9642</td>
<td>myo-Inositol</td>
<td>37.292</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.1768</td>
<td>Niacinamide</td>
<td>6.8987</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.9338</td>
<td>Pyridoxine.HCl</td>
<td>15.975</td>
</tr>
<tr>
<td>Histidine.HCl.H₂O</td>
<td>2.6242</td>
<td>Thiamine.HCl</td>
<td>4.3343</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.2092</td>
<td>d-Pantothenic acid</td>
<td>3.8724</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.1664</td>
<td>Vitamin B-12</td>
<td>1.3554</td>
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<tr>
<td>Lysine.HCl</td>
<td>7.1853</td>
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<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>1.8754</td>
<td><strong>Stock S-IV</strong></td>
<td>100X</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>Riboflavin</td>
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</tr>
<tr>
<td>Proline</td>
<td>1.151</td>
<td>Folic acid</td>
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<tr>
<td>Serine</td>
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<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>3.9541</td>
<td><strong>Stock S-V</strong></td>
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</tr>
<tr>
<td>Tryptophan</td>
<td>1.3008</td>
<td>CuSO₄.5H₂O</td>
<td>0.7491</td>
</tr>
<tr>
<td>Valine</td>
<td>4.0844</td>
<td>Na₂SeO₃</td>
<td>17.29</td>
</tr>
<tr>
<td>Glucose</td>
<td>96.48</td>
<td>ZnSO₄.7H₂O</td>
<td>201.25</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>2.772</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Individual Stock Solutions</strong></td>
<td></td>
</tr>
<tr>
<td>Ferric Citrate</td>
<td>61 g/L</td>
<td>Pluronic F-68</td>
<td>100 g/L</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>250</td>
<td>Lipoic Acid</td>
<td>1000</td>
</tr>
<tr>
<td>Ethanolamine.3HCl</td>
<td>3000</td>
<td>Glutathione (reduced)</td>
<td>500</td>
</tr>
<tr>
<td>Putrescine</td>
<td>160</td>
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<td></td>
</tr>
</tbody>
</table>

**Stock S-II**

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>17.416</td>
</tr>
<tr>
<td>Tyrosine.2Na.2H₂O</td>
<td>19.603</td>
</tr>
</tbody>
</table>

55
during storage.

3.3 T-flask Cultures

T-flasks (750 ml) were employed in batch and fed-batch cultures of hybridoma cells. Batch cultures were initiated at $1 - 2 \times 10^5$ cells/ml with 40 ml fresh IMDM supplemented with 5% dFBS, 4 mM glutamine, and 0.1% antibiotics in a 37 °C incubator with a 10% CO$_2$ overlay. Samples of 1 ml are taken daily and measured for viable and total cell densities with a Neubauer hemacytometer (Reichert, Buffalo, NY), and then centrifuged at 1000 RPM for at least 10 minutes. Supernatants were stored at -20 °C for analyses at a later time. In fed-batch cultures, a specifically designed initial medium, denoted Init-A (Table 3.1), was employed to avoid high lactate and ammonia production. After a short period of initial cell growth, a supplemental medium (Sup-1, Table 3.5) designed to meet the nutritional needs for cell growth was fed manually every 12 or 24 hours according to the rate of cell growth. Samples were taken every 12 or 24 hours. Cell enumeration was carried out immediately after the sample was taken. This was followed by the determination of the feeding volume and then the execution of the feeding protocol.

3.4 Spinner Flask Cultures

Two different sizes of spinner flasks, 150 ml and 200 ml, were employed in both batch and fed-batch cultures of CHO cells. Cultures were operated at an agitation rate of 150 rpm in a 37 °C incubator with a 5% CO$_2$ overlay. All CHO
cultures were inoculated at an initial cell density between 3 - 6 × 10^5 cells/ml. In batch cultures, JRH protein free medium supplemented with 4 mg/L insulin, 2 mg/L transferrin, and 2 g/L Primatone RL and initial medium Init-D supplemented with 4 mM glutamine, 20 mM glucose were employed. Samples were taken every 24 hours for cell counting. Cell-free samples were stored at -20 °C for subsequent analyses. In fed-batch cultures, a protein-free initial medium (Init-D, see Table 3.2) supplemented with 2 mg/L insulin was employed for the initiation of cell growth. Feeding of a supplemental medium (Table 3.5) designed using cell composition obtained from hybridoma CRL-1606 cells was executed manually every 12-24 hours from day one.

3.5 Bioreactor Cultures

3.5.1 Bioreactor Configuration and Control

A schematic figure of the bioreactor (Braun Biostat M, B. Braun) and control system for hybridoma cultures is shown in Figure 3.1. The total volume of the reactor is 2 liters with a maximum working volume of 1.7 liters. Two turbine impellers and a marine impeller were used for agitation. When the liquid volume is less than 1.3 liters, the top turbine impeller is not immersed in the liquid. Temperature control was achieved through a water jacket and a PT-100 temperature sensor. To avoid direct sparging, silicone tubing (SIP Medical grade Silastic Q7-4750 Silicone tubing, Baxter, McGaw Park, IL,) was wound on a steel cage inserted in the reactor for oxygen and carbon dioxide transport. The inlet gas, composed of oxygen, carbon dioxide, and nitrogen, passed
Figure 3.1 Schematic diagram of the fed-batch bioreactor and control system
through the silicone tubing immersed in the culture medium and the head space. Oxygen and carbon dioxide can diffuse through the tubing wall to provide oxygen and carbon dioxide for dissolved oxygen (DO) and pH control. Inlet gas composition was controlled by an ALERT computer via Mass Flow Controllers (Brooks 1000E), with a constant total gas flow rate of 200 ml/min. The supplemental medium was placed on an electronic balance (Mettle PE3000) in a 2-6 °C refrigerator. Signals from the balance were converted through a BCD Interface (Mettler CL 240), and inputted to the ALERT computer. The supplemental medium was pumped to the reactor by a peristaltic pump (on and off) through the control program which resided in a Compaq 286 monitor computer. Control strategies were developed through the program in the monitor computer and loaded into the ALERT controller computer. Dissolved oxygen and pH were monitored on-line using Ingold probes (Ingold, MA) and controlled at 60% of air saturation and 7.2, respectively, by adjusting the inlet oxygen and carbon dioxide compositions.

3.5.2 Bioreactor Operation

The bioreactor was first cleaned with tap water and rinsed thoroughly with deionized Milli-Q water. The pH probe was calibrated at pH 7.0 and pH 10.0 respectively. The bioreactor was filled with 1.5 liter phosphate balanced buffer solution before it was autoclaved for 1 hour. It was removed from the autoclave and allowed to cool before other installations were made. Three liquid tubing lines were employed for the medium feeding and the addition of base and acid. The tubing was connected to the reservoir when the bioreactor was installed.
Temperature was controlled at 37 °C during the DO calibration. The bioreactor was flushed with pure nitrogen overnight to attain a set-point of zero. Pure air is employed to saturate the liquid until a second stable reading was obtained and this value was adjusted to 100% of air saturation.

Both batch and fed-batch cultures were conducted using the 2-liter bioreactor with automatic process control. Cells obtained from spinner flasks were first centrifuged at 900 rpm for 5 minutes and the supernatant was carefully discarded. The cell pellet was then resuspended in IMDM for batch culture and in the initial medium (Init-C, Table 3.1) for fed-batch cultures. Before the cells and culture medium were added to the bioreactor, the PBS solution was completely removed using a vacuum pump. The fed-batch culture was initiated with a liquid volume of 900 ml and an initial cell density of $4 \times 10^5$ cells/ml. Automatic feeding of a supplemental medium (Sup-2) commenced right after the inoculation according to a strategy outlined in Chapter 5.

Samples range from 4 to 50 ml were taken every 12 hours to measure cell densities. For cell counting, 100 μl sample was diluted 2-17 fold depending on the cell density with 0.4% trypan blue solution (Sigma). The remaining sample was centrifuged at 1000 to 2000 rpm for 10 minutes. One milliliter of the cell-free supernatant was used for the measurement of osmolality, lactate, ammonia, glucose, phosphate, and magnesium. About 0.5 ml of the supernatant was deproteinated and stored separately with the rest of the cell-free supernatant at -20 °C for subsequent analyses. Concentrations of lactate, ammonia, glucose, phosphate, and magnesium were monitored off-line daily using a Biolyzer.
(Eastern Kodak Co.). The concentrations of lactate, ammonia, and glucose were confirmed by enzymatic assays (see analytic methods below) after the completion of the experiment. Ammonia concentration in the supplemental medium was also monitored every 2 - 3 days over the culture process using the Biolyzer.

3.6 Analytical Methods

3.6.1 Amino Acid Analysis

Amino acid concentrations were measured by derivatization with o-phthalaldehyde (OPA) followed by reverse phase high performance liquid chromatography (HPLC) using a Hypersil column (5µm, 100 × 2.1 mm id, Hewlett Packard, cat. no. 799160D-552). The OPA solution was prepared by dissolving 50 mg in 1 ml absolute methanol. Forty µl mercaptoethanol was added, and diluted to 10 ml with borate buffer (12.36 g boric acid in 1 liter water, pH 10.4, filtered). Finally, 10 µl of 30% aqueous brij 35 surfactant was added. Two solvents were employed. Solvent A was composed of 0.05M Na2HPO4, 0.05M NaOAc, 2% v/v methanol, 2% v/v tetrahydrofuran, pH 7.5. Solvent B was prepared with 650 ml methanol in 350 ml water. Amino acid elution was performed using a flow rate of 0.5 ml/min with the following gradient program: 2 min, 18% B; 8 min, 25% B; 12 min, 40% B; 15 min, 55% B; 21 min, 75% B; 24 min, 100% B; 25 min, 0% B. Samples were deproteinated prior to the assay. To deproteinate a sample, 150 µl 20% (w/v) trichloroacetic acid was added to 510 µl of the supernatant, and centrifuged in a 1.5 ml centrifuge tube at 10,000 rpm
for 10 min using a Biofuge 13 centrifuge (Baxter Scientific), followed by the addition of 170 μl 25% (w/v) KHCO₃ to 0.5 ml of the supernatant. Eight μl sample was mixed with 4 μl OPA for 3 minutes to allow the derivatization reaction to complete before injection into the column.

3.6.2 Enzymatic Analysis for Glucose, Lactate, and Ammonia

Glucose was analyzed by assay kits from Sigma Chemical Co. (cat. no. 16-20), based on enzymatic reactions using hexokinase and glucose-6-phosphate. The lactate assay was based on the reaction of lactate dehydrogenase. Ammonia was measured through enzymatic reaction with glutamate dehydrogenase. Samples for the glucose and lactate assays were deproteinated and neutralized prior to each assay. Cells in samples for ammonia analysis were centrifuged for 10 minutes at 10,000 rpm with a Biofuge-A microcentrifuge (American Scientific Products). Absorbance was measured at a wavelength of 340 nm with a Perkin-Elmer Lambda-3 spectrophotometer (Norwalk, CT).

3.6.3 Monoclonal Antibody Analysis

An enzyme-linked immunosorption assay (ELISA) was employed to determine the titer of the monoclonal anti-fibronectin antibody using cell-free samples. Kappa mouse IgG1 (Sigma, cat. no. M9269) was used as a standard. First, 100 μl antigen solution (antibody to mouse IgG1, Sigma, cat. no. M 8770) was placed in a 96 well microtiter plate and incubated for 1 hour. After rinsing with 3 × 100 μl washing buffer (2.5 ml amps tween 20, 5 ml blocker BSA in PBS and 1 pack of BupH Dulbecco-PBS to 500 ml Milli-Q water, Sigma), 200 μl blocking
buffer (10 ml Blocker BSA in PBS in 100 ml BupH Dulbecco-PBS, Sigma) was added and incubated for another 30 minutes followed by washing with buffer. One hundred μl sample or standard solution was added to each well and incubated for 1 hour. After rinsing with 3 × 100 μl washing buffer, 100 μl labeled secondary antibody was added and incubated for 30 minutes. The plate was then rinsed with 3 × 100 μl washing buffer and incubated with 100 μl washing buffer for another 5 minutes. Then 100 μl of enzyme substrate solution (ABTS substrate, Pierce cat. no. 37615) was added and incubated for 30 minutes. Absorbance was measured at a wavelength of 405 nm via a kinetic microplate reader (Molecular Devices Corporation, Palo Alto, CA). Samples were diluted 100-12000 fold with blocking buffer prior to assay depending on the antibody concentrations.

3.6.4 Dry Cell Weight Measurement

A 50 ml sample, containing about 10^8 cells, was placed into a 50 ml centrifuge tube (Corning, NY) and centrifuged at 1500 rpm for 10 min with an IEC Centra-4B centrifuge (International Equipment Co.). Total cell density was determined using four samples with a Neubauer hemacytometer (Reichert, Buffalo, NY). Cell pellet was then washed twice with 10 ml PBS and centrifuged. Supernatant was carefully discarded. The pellet was then transferred into a pre-weighted pan and dried at 60 °C to a constant weight. Three to five parallel measurements were carried out to obtain reliable average results.
3.6.5 Protein Assay

Biuret assay (Read, 1984; Packer, 1967) was employed to determine the cellular protein content. Samples containing about 10^7 cells were placed into a 15 ml centrifuge (Corning, NY) tube and were prepared the same way as for dry cell weight measurement. Cells were disrupted in 0.5 ml lysis buffer (0.5% Triton X-100, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) at 4 °C for 40 min. After cell lysis, 1.5 ml Biuret reagent (1.5 g/L CuSO_4·5H_2O, 6 g/L sodium potassium tartrate and 30 g/L NaOH) was added and mixed thoroughly. After a 30 min incubation at room temperature, absorbance was measured at 500 nm using a Perkin-Elmer Lambda-3 spectrophotometer (Norwalk, CT). A series of standards with concentrations from 1 to 10 mg/ml were prepared using bovine serum albumin (Sigma). This assay was conducted in triplicate.

3.6.6 Carbohydrate Assay

Total cellular carbohydrates were determined by the phenol reaction method (Hanson and Philips, 1981). The cell pellet was prepared the same way as in the protein assay. Cells were disrupted with 0.5 ml lysis buffer in a 15 ml centrifuge tube, and then transferred into a 20 ml thick-walled Pyrex test tube. The centrifuge tube was washed with another 0.5 ml lysis buffer and was combined with the liquid in the test tube. One ml phenol reagent (50 g/L aq.) was added and mixed rapidly and thoroughly with the sample. Finally, 5 ml concentrated sulfuric acid was added and rapidly mixed. After a 30 min of incubation at room temperature, absorbance was measured at 488 nm using a Perkin-Elmer Lambda-3 spectrophotometer (Norwalk, CT). A series of
standards with concentrations from 10 to 100 μg/ml were prepared using glucose. Triplicate measurements were needed for accurate result.

3.6.7 Lipid Extraction and Purification

The extraction of total cellular lipids was carried out using the methods of Folch et al. (Bligh and Dyer, 1959; Folch at al., 1956; Gerschenson et al., 1967; Nelson, 1975; Packer, 1967; Stein and Smith, 1982). Samples containing about 3 × 10^8 cells (150 ml) were centrifuged in three 50 ml centrifuge tubes the same way as in the dry cell weight assay. Pellets were washed with 10 ml PBS and combined into one test tube. After centrifugation, the supernatant was discarded, and 0.5 ml Milli-Q water was added and mixed. A total of 10 ml methanol was mixed with the cells and the mixture was transferred quickly and completely into a 50 ml glass cylinder. Then 20 ml chloroform was added and mixed. After 40 min of extraction, the mixture was filtered through a 0.8 micron filter paper (MSI, Westboro, MA). The filter paper was washed with 5 ml methanol and 10 ml chloroform. Filtrate was collected in a graduated cylinder and 8 ml NaCl (w/v 0.9% aq.) was added. The mixture was allowed to separate into two phases. After removing the upper phase, 13 ml of Chloroform/methanol/0.9% aq. NaCl (3:48:47) was added and mixed. The upper phase was carefully discarded. This step was repeated twice. The final lower phase contained lipids and was dried in a weighing pan at 40 °C in a vacuum desiccator to a constant weight. Duplicate measurements were conducted.
3.6.8 DNA and RNA Assay

The cellular DNA and RNA contents were determined by the Diphenylamine and Orcinol reactions respectively. Samples containing about $2 \times 10^7$ cells were prepared and disrupted the same manner as in the protein assay. The procedure for DNA and RNA extraction has been described elsewhere (Hanson and Philips, 1981). A DNA standard was prepared using deoxyribose with concentrations ranging from 10 to 50 $\mu$g ml$^{-1}$. A adenosine 5'-monophosphate solution with concentrations from 20 to 100 $\mu$g ml$^{-1}$, was used as a standard for the RNA assay.

3.6.9 Amino Acid Composition in Cellular Proteins

The analysis of the amino acid composition of cellular proteins was performed by Analytical Biotechnology Services (Boston, MA). Hydrolysis was conducted with 6N HCl at 110°C for 24 hours. Following hydrolysis, the sample was vacuum dried, and placed in a re-drying solution (ethanol:water:triethylamine; 2:2:1) and vacuum dried again. The hydrolysate was added to ethanol, water, and triethylamine (7:1:1) and derivatized by adding phenylisothiocyanate. The amino acids were analyzed using a Waters PicoTag HPLC system. The concentrations for aspartate/asparagine and glutamate/glutamine were reported together. Average values from mammalian cell line for asparagine and glutamate were used to calculate the values for aspartate and glutamine. Samples containing $10^6$ cells were centrifuged and washed twice with 1 ml PBS. Supernatant was completely discarded. Finally, 0.5 ml PBS was added to each sample, and shipped in dry ice for analysis.
3.6.10 Other Assays

Osmolality was measured using a µOSMETTE (model 5004, Precision Systems Inc. MA) with 50 µl cell free sample. Lactate, ammonia, glucose, phosphate, and magnesium were monitored off-line in the bioreactor cultures using a Biolyzer (Eastman Kodak Co.). Medium pH was measured with an Inglod pH meter. The concentration of interferon-γ was determined with an ELISA kits purchased from Endogen Inc. (Cat. # EH-IFNG, Cambridge, MA).

3.7 Calculations

The production of lactate, ammonia, and product during cell cultivation between the \( (n-1) \) and \( nth \) samples was calculated using the following material balances:

\[
P_{\text{lac, } n} = C_{\text{lac, } n} (V_n + V_{s, n}) - C_{\text{lac, } n-1} V_{n-1} \tag{3.1}
\]

\[
P_{\text{amm, } n} = C_{\text{amm, } n} (V_n + V_{s, n}) - C_{\text{amm, } n-1} V_{n-1} - C_{\text{amm}}^S \Delta V_{F, n} \tag{3.2}
\]

\[
P_{\text{p, } n} = C_{\text{p, } n} (V_n + V_{s, n}) - C_{\text{p, } n-1} V_{n-1} \tag{3.3}
\]

The average specific production rates of lactate, ammonia, and product were defined by equation (3.4).

\[
\bar{q}_{i} = \frac{1}{\tau} \sum_{n=1}^{m} P_{i, n} \quad (i = \text{lac, amm, or p}) \tag{3.4}
\]

where \( \tau \) is the integral of viable cell number over the culture time:

\[
\tau = \int_{0}^{t_{m}} N_v \, dt = \sum_{n=1}^{m} (N_{v, n} + N_{v, n-1} - X_{v, n-1} V_{s, n-1}) (t_n - t_{n-1})/2 \tag{3.5}
\]
The amount of each nutrient consumed between the \((n-1)\) and \(n\)th samples was calculated using mass balances:

\[
\delta_{k,n} = C_{k,n-1} V_{n-1} + \Delta V_{F,n} C_{k}^{s} - C_{k,n} V_{n} \quad (k = \text{glc, amino acids}) \tag{3.6}
\]

The total consumption of the \(k\)th nutrient was then:

\[
\delta_{k} = \sum_{n=1}^{m} \delta_{k,n} \tag{3.7}
\]

The total number of cells produced before the \(n\)th sample was calculated from equation (3.8).

\[
\Delta N_{t,n} = N_{t,n} + \sum_{j=1}^{n-1} X_{t,j} V_{s,j} - N_{t,0} \tag{3.8}
\]

The molar ratios of byproducts over total number of cells produced were determined from equation (3.9):

\[
Y_{i/cell} = \frac{1}{\Delta N_{t,m}} \sum_{n=1}^{m} P_{i,n} \quad (i = \text{lac or amm}) \tag{3.9}
\]

By definition, the ratios of byproducts over nutrients consumed \((Y)\) were calculated from the following equations:

\[
Y_{\text{lac/glc}} = \frac{\sum_{n=1}^{m} P_{\text{lac},n}}{\sum_{n=1}^{m} \delta_{\text{glc},n}} \tag{3.10}
\]

\[
Y_{\text{amm/gln}} = \frac{\sum_{n=1}^{m} P_{\text{amm},n}}{\sum_{n=1}^{m} \delta_{\text{gln},n}} \tag{3.11}
\]
The accumulative concentration of the kth nutrient added was calculated from equation (3.12).

\[
C_k^a = \sum_{n=1}^{m} \frac{C_k^a \Delta V_{F,n}}{V_n}
\]

(3.12)

The average specific growth rate was calculated by dividing the number of cells produced by the time integral of viable cells.

\[
\bar{\mu} = \frac{\Delta N_t}{\tau}
\]

(3.13)

In the fed-batch system, because of the volume change caused by sampling and feeding, the cell growth kinetics was expressed by cell numbers instead of cell density as one usually finds in literature. The following equations were employed to calculate the specific death rate and growth rate using cell density measurements from two subsequent samples. These values were then employed to project the viable and total cell numbers in the period between current and next samples.

\[
V_n = V_{n-1} + \Delta V_{F,n} - V_{s,n}
\]

(3.14)

\[
N_{v,n} = X_{v,n} \left( V_n + V_{s,n} \right)
\]

(3.15)

\[
N_{t,n} = X_{t,n} \left( V_n + V_{s,n} \right)
\]

(3.16)

\[
\alpha = \frac{\ln \left( N_{v,n}/N_{v,n-1} \right) \left[ \left( N_{t,n} - N_{v,n} \right) - \left( N_{t,n-1} - N_{v,n-1} \right) \right]}{\left( t_n - t_{n-1} \right) \left( N_{v,n} - N_{v,n-1} \right)}
\]

(3.17)

\[
\mu = \alpha + \frac{\ln \left( N_{v,n}/N_{v,n-1} \right)}{t_n - t_{n-1}}
\]

(3.18)
\[ N_v = N_{v,n} e^{(\mu - \alpha)(t - t_n)} \]  

(3.19)

\[ N_l = N_{l,n} + \frac{\mu N_{v,n}}{\mu - \alpha} \left[ e^{(\mu - \alpha)(t - t_n)} - 1 \right] \]  

(3.20)
4. Stoichiometric Equation Governing Animal Cell Growth

4.1 Introduction

Animal cell growth is a complicated process where the metabolic reaction network consists of hundreds of different reactions. Even though the stoichiometry for each reaction in the metabolic network is known, the overall stoichiometry for the cell growth process is unknown unless extents for all of the reactions are defined. The production of cell mass is a necessary prerequisite to produce the desired protein which is synthesized by viable cells. In addition, there are several major by-products: carbon dioxide, lactate, ammonia, and some non-essential amino acids. These factors (multiple products and by-products) further complicate the problem.

The synthesis of cell mass and product requires nutrients such as glucose, amino acids, vitamins, inorganic salts, and serum components. Animal cell mass contains a very small fraction of inorganic salts, and these compounds are abundant in the conventional media. Thus, the chance of any stoichiometric limitation of inorganic salts is unlikely. Inorganic salts are therefore not considered in the stoichiometric equation. The essential roles of serum are to provide growth factors, hormones, and binding proteins and to protect cells from hydrodynamic stress (Iscove and Melchers, 1978; Jayme and Blackman, 1985; Keen and Rapson, 1995; Zhou and Hu, 1995). Small molecules present in
serum, such as amino acids, glucose, vitamins, and fatty acids can be replaced by nutrients in the basal medium. Essential components in serum are assumed to be sufficient for cell growth and are not considered in the stoichiometric equation.

The purpose of studying the stoichiometric equation for animal cell growth is to determine the nutritional requirement for cell growth. This is critical to balance the consumption and requirement of nutrients for optimal cell growth. The goal is to correlate the nutritional requirement with the production of cell mass. This will allow one to determine the demands for nutrients according to cell growth. However, unlike a single reaction where the stoichiometry is defined by simple element balances, the overall stoichiometry for cell growth is undefined due to factors such as by-product formation and hence is affected by the nutritional environment. For example, the molar ratio of lactate production to glucose consumption increases with glucose concentration during the growth phase. Due to the low efficiency of glucose utilization for energy production at high glucose level, the amount of glucose required for the production of the same amount of cell mass is greater than that at a low glucose level.

In a traditional batch culture, the consumption of nutrients and the production of cell mass, product, and by-products can be determined experimentally. These data are sufficient to determine the overall stoichiometric equation for cell growth. Certainly, this approach is cell type and culture condition specific and hence is not applicable to other conditions. Especially, if information obtained
from such an inefficient process is employed to design a new process, improvement in culture performance is unlikely to be achieved.

A two step approach is taken in this thesis to determine the stoichiometric equation under conditions that lead to optimal cell growth. First, the stoichiometric equation for a hypothetical ideal system is determined. On the basis of the ideal system, the stoichiometric equation for a real system is determined by applying process optimizing criteria. The details are presented below.

4.2 Stoichiometric Equation for an Ideal System

4.2.1 Definition of Ideal System

An ideal system is defined as a system where no lactate, ammonia, or non-essential amino acid is produced which assumes that efficiencies of glucose and glutamine utilization are at their maximum values. The requirements of cell growth for non-essential amino acids are provided from extracellular sources instead of the biosynthesis from glutamine and glucose.

4.2.2 Stoichiometric Equation

As described above, the biosyntheses of cell mass and product require many essential nutrients. However, the focus of this thesis is on glucose, amino acids, and vitamins. The studies on salts, growth factors, oxygen, etc. are outside the
scope of this thesis. Hence, for simplicity, these nutrients and carbon dioxide are not shown in the overall stoichiometric equation.

In the ideal system, the overall stoichiometric equation for cell mass and product synthesis becomes:

\[
\theta_{\text{glc}} \text{[Glucose]} + \sum_{i=1}^{20} \theta_{a,i} \text{[Amino Acids]} + \sum_{j=1}^{10} \theta_{v,j} \text{[Vitamin]}_j \\
\rightarrow \text{[Cell Mass]} + \theta_p \text{[Product]} + \theta_{\text{ATP}} \text{[ATP]}
\]  

(4.1)

It should be noted that the ATP term included in the stoichiometric equation is not a net production of ATP but the amount required for the synthesis of cell mass and product. The stoichiometric coefficient for nutrient is defined as the amount of nutrient consumed per cell produced. The stoichiometric coefficient for cell mass is therefore normalized to be unity. The stoichiometric coefficient for product is defined as the amount of product synthesis normalized by the increase of the total number of cells. Similarly, the stoichiometric coefficient for ATP is defined as the amount of ATP required for cell mass and product formation per cell.

In the ideal system, the stoichiometric coefficient for each nutrient is assumed to be the hypothetical minimum value because there is no by-product formation. Consequently, nutrient efficiency is assumed to be its hypothetical maximum value. The word "hypothetical" indicates that these values are not achievable in a real cell culture process. The stoichiometric coefficient in the ideal system is
independent of the culture process and is then bounded completely by the material and energy balances.

Even though equation (4.1) appears simple, the determination of the unknown stoichiometric coefficients is not an easy task. There are 33 unknown variables with only three chemical element balances available. Therefore, the equation cannot be solved by traditional elemental balances without considering the detailed metabolic reaction network governing the biosyntheses of cell mass, product, and energy.

4.2.3 Metabolic Reaction Network

Cellular components of animal cells consist of a complex mixture of cellular proteins, lipids, carbohydrates, DNA, RNA, water, and inorganic salts (Campbell, 1990; Darnell et al., 1990). Since inorganic salts are neglected in the stoichiometric equation, water and inorganic salts will not be considered in cell mass. There are hundreds of different reactions involved in animal cell metabolism. Fortunately, not all of these reactions are relevant to the overall stoichiometric equation. In this section, the metabolic reaction network involved in the biosyntheses of cellular protein, DNA, RNA, lipids, carbohydrates, product, energy, and reducing power is discussed.

The metabolic reaction network for the ideal system consists of three major parts: protein synthesis, nucleotide synthesis, and glucose metabolism. This network is relatively simple as compared to the network for a real system,
mainly because glutamine metabolism is simplified. The simplification that no ammonia is produced in the ideal system assumes that glutamine is not used for energy or non-essential amino acid production. This is because energy production from glutamine is associated with ammonia and non-essential amino acid biosynthesis.

4.2.3.1 Protein Synthesis

The synthesis of proteins in cells and product requires all of the twenty amino acids. Cellular proteins are a mixture of enzymes, membrane proteins, structural proteins, etc. The amino acid sequences for most of these proteins in cells are unknown. To simplify this problem, the synthesis of the protein mixture in the cells is assumed to be represented by a single equation:

\[
\sum_{i=1}^{20} \theta_{a,i}^{cp} [\text{Amino Acids}]_i = \text{Proteins} \tag{4.2}
\]

The stoichiometric coefficients for the amino acids in equation (4.2), \( \theta_{a,i}^{cp} \), are thus the overall amino acid composition of the cellular proteins and could be measured experimentally.

The stoichiometric equation describing product synthesis can be written in a similar fashion if the amino acid sequence of the product is known.

\[
\sum_{i=1}^{20} \theta_{a,i}^{cp} [\text{Amino Acids}]_i = \text{Product} \tag{4.3}
\]

In case where the amino acid sequence of the product is not known, the amino acid composition of the product can be determined through purification and protein characterization. When the amino acid composition of the product is
unavailable, the amino acid composition of the product protein can be assumed to be the same as the overall cellular protein composition without encountering significant errors.

4.2.3.2 Nucleotide Synthesis

In DNA and RNA biosyntheses, eight different nucleotides need to be synthesized before the DNA and RNA polymerization occurs. The pathway for the synthesis of each nucleotide is quite complicated (Kelly, 1972; Stryer, 1988). Glucose is the carbon source for the formation of the ribose in RNA and deoxyribose in DNA through the pentose phosphate cycle. Five amino acids, namely glutamine, aspartate, serine, glutamate, and glycine, are involved in the synthesis of purine and primidone. It is assumed that the conversion of serine to glycine provides the one carbon unit required for the synthesis of nucleotides. The overall reactions (not balanced for simplicity) involved in the synthesis of these nucleotides are shown below:

\[
\text{Gln} + \text{Asp} + \text{Ribose-5-P} + 5\text{ATP} + \text{NAD}^+ \rightarrow \text{UTP} + \text{AMP} + 4\text{ADP} + \text{NADH} + \text{Glu} \quad (4.4)
\]
\[
2\text{Gln} + \text{Asp} + \text{Ribose-5-P} + 6\text{ATP} + \text{NAD}^+ \rightarrow \text{CTP} + \text{AMP} + 5\text{ADP} + \text{NADH} + 2\text{Glu} \quad (4.5)
\]
\[
2\text{Gln} + 2\text{Asp} + 2\text{Ser} + \text{Ribose-5-P} + \text{CO}_2 + 7\text{ATP} + 2\text{NAPD}^+ \rightarrow \\
\text{ATP} + \text{AMP} + 6\text{ADP} + 2\text{NADPH} + 2\text{Fumarate} + \text{Gly} + 2\text{Glu} \quad (4.6)
\]
\[
3\text{Gln} + \text{Asp} + 2\text{Ser} + \text{Ribose-5-P} + \text{CO}_2 + 7\text{ATP} + 2\text{NAPD}^+ + \text{NAD}^+ \rightarrow \\
\text{GTP} + 2\text{AMP} + 5\text{ADP} + 2\text{NADPH} + \text{NADH} + \text{Fumarate} + 3\text{Glu} + \text{Gly} \quad (4.7)
\]
\[
2\text{Gln} + \text{Asp} + \text{Ribose-5-P} + 6\text{ATP} + \text{NADPH} + \text{NAD}^+ \rightarrow 
\]

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\[
dCTP + AMP + 5ADP + NADH + 2Glu + NADP^+ \quad (4.8)
\]
\[
2Gln + Asp + Ribose-5-P + Ser + 7ATP + 2NADPH + NAD^- \rightarrow
dTTP + AMP + 6ADP + NADH + 2NADP^+ + 2Glu + Gly + NH_3 \quad (4.9)
\]
\[
2Gln + 2Asp + 2Ser + Ribose-5-P + CO_2 + 6ATP + NAPDH\rightarrow
dATP + AMP + 5ADP + NADPH + 2Fumarate + Gly + 2Glu \quad (4.10)
\]
\[
3Gln + Asp + 2Ser + Ribose-5-P + CO_2 + 7ATP + NAPD^- + NAD^- \rightarrow
dGTP + 2AMP + 5ADP + NADPH + NADH + Fumarate + 3Glu + Glu \quad (4.11)
\]

### 4.2.3.3 Glucose Metabolism

Glucose metabolism plays a very critical role in animal cell growth as shown in Figure 4.1. Five independent reactions are taken into consideration, and these are the production of ribose, carbohydrates, lipids, NADPH, and pyruvate. Each of these reactions is actually a set of serial reactions, and some reactions between different sets are interrelated. By lumping these serial reactions together, the original reaction network is significantly simplified, and the number of reactions is dramatically reduced. It should be noted that the lumping approach does not affect the stoichiometry for cell metabolism. More details are presented below.

Ribose is the common sugar source for the synthesis of both ribonucleotides in RNA and deoxyribonucleotides in DNA. Since ribose is not an essential nutrient for cell growth, it is produced from glucose via the pentose phosphate cycle as
Figure 4.1 Glucose metabolism in an ideal system for animal cell growth
shown by eq. (4.12).
\[
\text{Glucose} + \text{ATP} + 2 \text{NADP}^+ \rightarrow \text{Ribose-5-P} + 2 \text{NADPH} + \text{ADP} + \text{CO}_2 
\] (4.12)

Reducing power in the form of NADPH is required in many synthetic reactions such as the reduction of ribonucleotides to deoxyribonucleotides. A certain amount of this NADPH requirement can be met by the accompanied NADPH formation in other metabolic reactions. The rest of the requirement is satisfied by the consumption of glucose in the pentose cycle for NADPH production. The overall stoichiometry for the conversion is shown by eq. (4.13).
\[
\text{Glucose} + \text{ATP} + 12 \text{NADP}^+ \rightarrow 12 \text{NADPH} + \text{ADP} + 6 \text{CO}_2 
\] (4.13)

Cellular carbohydrates are a complicated mixture of sugars and their polymers. Most of the sugars are inter-convertible with glucose and are derived directly from the pentose phosphate cycle of glucose metabolism. Carbohydrates have a similar overall chemical composition and can be characterized as \((\text{CH}_2\text{O})_n\). For simplicity, glycogen is assumed as the major carbohydrates in cells, and hence the stoichiometric equation for carbohydrate synthesis becomes:
\[
\text{Glucose} + \text{Glycogen}_n + 2 \text{ATP} \rightarrow \text{Glycogen}_{n+1} + 2 \text{ADP} 
\] (4.14)

Cellular lipids are a mixture of fats, phospholipids, and steroids (Darnell et al., 1990). Both phospholipids and steroids are important components of the animal cell membranes. Fats and phospholipids are synthesized from two major precursors, glycerol and fatty acids. Glycerol is derived mainly from the intermediates of the glycolytic pathway and the pentose phosphate cycle. Fatty
acids can be utilized from the culture medium or it can be synthesized from acetyl-CoA (Grammatikos et al., 1994). Cholesterol, which is one of the most abundant steroids, is an important component of the animal cell membranes as well as a precursor for other steroids. Cholesterol is also synthesized via acetyl-CoA. Hence, steroids are assumed to be derived from acetyl-CoA, either directly or indirectly. Acetyl-CoA can be derived from pyruvate which is an important substrate for lipid synthesis and for the TCA cycle. Since no glutamine is assumed to enter the TCA cycle for energy production, pyruvate is assumed to be derived solely from glucose in the ideal system.

The detailed pathway for the fatty acid synthesis from glucose is shown in Figure 4.2. Glycerol-3-phosphate (G3P) can be incorporated into phospholipids. However, because G3P contributes only three carbons to phospholipid which contains more than 40 carbons, this pathway is simplified and lumped into the overall fatty acid biosynthesis pathway. The overall stoichiometric equation for lipid synthesis is then simplified to be:

\[
\text{Glucose} + 4 \text{ ATP} + 2 \text{ NADPH} + 2 \text{ NAD}^+ + (\text{Fatty Acids})_{2n} \rightarrow
\]

\[
(F\text{atty Acids})_{2n+2} + 2 \text{ NADH} + 4\text{ADP} + 2\text{NADP}^+ + 2\text{CO}_2
\] (4.15)

In the ideal system, glucose is assumed to be the sole source for energy production. This assumption simplifies the TCA cycle since only pyruvate is allowed to enter the cycle. The energy metabolism is further simplified by the assumption that no lactate is produced. The stoichiometric equation for the conversion of glucose to carbon dioxide through the glycolytic pathway and the
Figure 4.2 Biosynthesis pathway for fatty acid formation from glucose
TCA is shown by equations (4.16) and (4.17).

\[
\text{Glucose } + 2\text{ADP} + 2\text{NAD}^+ \rightarrow 2\text{Pyruvate} + 2\text{ATP} + 2\text{NADH} \quad (4.16)
\]

\[
\text{Pyruvate} + 4\text{NAD} + \text{FAD} + \text{GDP} \rightarrow 3\text{CO}_2 + 4\text{NADH} + \text{FADH}_2 + \text{GTP} \quad (4.17)
\]

### 4.2.4 Determination of the Stoichiometric Coefficients

In the ideal system, the stoichiometric equation for cell metabolism is defined by cell composition, dry cell weight, product formation, and energy demand. The average dry cell composition (dry weight percentage) is composed of the following: proteins \((Z_p)\), DNA \((Z_D)\), RNA \((Z_R)\), lipids \((Z_L)\), and carbohydrates \((Z_C)\). The average total dry cell weight is denoted \(W\) (mg/cell).

#### 4.2.4.1 Stoichiometric Coefficient for Product

The cell growth kinetics can be expressed by equations (4.18), (4.19), and (4.20).

\[
\frac{dX_v}{dt} = (\mu - \alpha) X_v \quad (4.18)
\]

\[
\frac{dX}{dt} = \alpha X_v \quad (4.19)
\]

\[
\frac{dX_l}{dt} = \mu X_v \quad (4.20)
\]

The product synthesis rate can be expressed by equation (4.21).

\[
\frac{dP}{dt} = q_p X_v \quad (4.21)
\]

By definition, the stoichiometric coefficient for product can be derived from eqs. (4.20) and (4.21):
\[ \theta_d = \frac{\text{amount of product produced}}{\text{number of cells produced}} = \frac{\text{dP}}{\text{d}X_t} = \frac{q_p}{\mu} \]  

(4.22)

### 4.2.4.2 Stoichiometric Coefficient for ATP

Based upon the assumption that dead cells do not consume ATP, the ATP consumption rate can be defined in a similar way as the product formation rate by using a specific ATP consumption rate as shown by equation (4.23).

\[ \frac{\text{d}[\text{ATP}]}{\text{dt}} = q_{\text{ATP}} X_v \]  

(4.23)

According to a maintenance model, ATP is required for the synthesis of macromolecules and also for cell maintenance (Paul, 1965; Stouthamer, 1973, 1979). The specific ATP consumption rate is thus defined as:

\[ q_{\text{ATP}} = \frac{\mu}{\sqrt{v_{\text{ATP}}}} + m_{\text{ATP}} \]  

(4.24)

The stoichiometric coefficient for ATP can then be derived according to its definition as:

\[ \theta_{\text{ATP}} = \frac{\text{amount of ATP produced}}{\text{number of cells produced}} = \frac{\text{d}[\text{ATP}]}{\text{d}X_t} = \frac{q_{\text{ATP}}}{\mu} \]  

(4.25)

### 4.2.4.3 Stoichiometric Coefficients for Amino Acids

The total moles of nucleotides in DNA (\(N_{\text{DNA}}\)) and RNA (\(N_{\text{RNA}}\)) can be calculated from the cell composition and the average molecular weights of DNA and RNA. The number of moles of each base per cell is then calculated by multiplying the total moles of nucleotides by the base compositions of DNA and RNA.
\[ N_{\text{DNA}} = Z_{\text{D}} W / M_{\text{D}} \quad (4.26) \]
\[ N_{\text{RNA}} = Z_{\text{R}} W / M_{\text{R}} \quad (4.27) \]
\[ N_A = N_{\text{DNA}} D_A + N_{\text{RNA}} R_A \quad (4.28) \]
\[ N_G = N_{\text{DNA}} D_G + N_{\text{RNA}} R_G \quad (4.29) \]
\[ N_C = N_{\text{DNA}} D_C + N_{\text{RNA}} R_C \quad (4.30) \]
\[ N_T = N_{\text{DNA}} D_T \quad (4.31) \]
\[ N_U = N_{\text{RNA}} D_U \quad (4.32) \]

where \( M_{\text{D}} \) and \( M_{\text{R}} \) are the average molecular weight of the nucleotides in DNA and RNA, respectively.

\[ M_D = M_{\text{PO}_4} + M_{C_5H_7O} + D_A M_{C_9H_{14}N_5} + D_G M_{C_5H_4ON_9} \]
\[ + D_C M_{C_9H_4ON_9} + D_T M_{C_9H_5O_2N_2} \quad (4.33) \]
\[ M_R = M_{\text{PO}_4} + M_{C_5H_7O} + R_A M_{C_9H_{14}N_5} + R_G M_{C_5H_4ON_9} \]
\[ + R_C M_{C_9H_4ON_9} + R_U M_{C_4H_3O_2N_2} \quad (4.34) \]

The stoichiometric requirements for glutamine, aspartate, serine, glutamate, and glycine for the synthesis of DNA and RNA can be calculated according to the stoichiometric equations. This is done by multiplying the moles of each base by the stoichiometric coefficient for the corresponding amino acid in equations (4.4) through (4.11). A minus sign signifies a net production instead of a net consumption.

\[ \theta_{\text{nuc}}^{\text{gin}} = 3 N_G + 2[N_A + N_C + N_T] + N_U \quad (4.35) \]
\[ \theta_{\text{nuc}}^{\text{asp}} = N_{\text{DNA}} + N_{\text{RNA}} \quad (4.36) \]
\[ \theta_{\text{ser}}^{\text{nuc}} = 2[N_A + N_d] + N_r \]  
(4.37)

\[ \theta_{\text{glu}}^{\text{nuc}} = -\theta_{\text{gin}}^{\text{nuc}} \]  
(4.38)

\[ \theta_{\text{giv}}^{\text{nuc}} = -[N_A + N_d + N_r] \]  
(4.39)

All of the twenty amino acids are needed for the synthesis of cellular proteins and product as shown by eqs. (4.2) and (4.3). The molar percentages of amino acids in cellular proteins \( (O_i^{\text{op}}) \) and product \( (O_i^{pp}) \) can be experimentally determined. The average molecular weights of peptides in cellular proteins \( (M_p^{op}) \) and in product \( (M_p^{pp}) \) are determined by equations (4.40) and (4.41), respectively.

\[ M_p^{op} = \sum_{i=1}^{20} \frac{M_{a_i} O_i^{op}}{100} \]  
(4.40)

\[ M_p^{pp} = \sum_{i=1}^{20} \frac{M_{a_i} O_i^{pp}}{100} \]  
(4.41)

The stoichiometric requirement of each amino acid for protein synthesis can be determined by the cellular protein content and the amount of product synthesized.

\[ \theta_i^p = \left( \frac{Z_p W}{M_p^{op}} \right) \frac{O_i^{op}}{100} + \left( \frac{\theta_i}{M_p^{pp}} \right) \frac{O_i^{pp}}{100} \]  
(for 20 amino acids)  
(4.42)

The stoichiometric coefficient of each amino acid is then the combination of the requirements for protein, DNA, and RNA syntheses as shown by equation (4.43).
\[ \theta_{a,i} = \theta_{a,i}^p + \theta_{a,i}^{nuc} \]  

(4.43)

4.2.4.4 Stoichiometric Coefficient of Glucose

Glucose is used for the syntheses of lipids, carbohydrates, pentoses in DNA and RNA, and ATP production. It is assumed in the following calculation that glucose is the sole source for cellular carbohydrates and lipids. Hence, the possible uptake of free fatty acids from culture medium is neglected. This is quite reasonable as shown by the small requirement of free fatty acids in protein free media (Kobayashi et al. 1994). The determination of the stoichiometric coefficient of glucose requires the predetermination of the reaction extents of glucose metabolism (Figure 4.1).

The glucose requirement for carbohydrate is derived from a carbon balance. One mole glucose provides six moles of carbon. The total moles of carbon in cellular carbohydrates per cell is the weight of carbohydrates per cell, \( Z_c W \), divided by the molecular weight of the carbohydrate unit \( \text{CH}_2\text{O} \). The stoichiometric demand for glucose in carbohydrate synthesis becomes:

\[ F_{18} = \frac{Z_c W}{[M_{\text{CH}_2\text{O}} \times 6]} \]  

(4.44)

Similarly, the stoichiometric demand for glucose in lipid synthesis is derived from a carbon balance as described by equation (4.45).

\[ F_{19} = \frac{Z_c W}{[M_{\text{CH}_2} \times 4]} \]  

(4.45)
The number of moles of ribose-5-phosphate required for DNA and RNA synthesis equals that of the total nucleotides in DNA and RNA as defined by equations (4.26) and (4.27), respectively. According to equation (4.12), the formation of one mole ribose-5-phosphate uses one mole glucose. The stoichiometric requirement for glucose in DNA and RNA synthesis is then:

\[ F_{17} = N_{DNA} + N_{RNA} \]  

(4.46)

Reducing power (NADPH) is required for the reductive syntheses of lipids and nucleotides. The need for NADPH in the reparation of lipid membranes due to peroxidation is neglected due to difficulties to estimate the stoichiometric demand. The total amount of NADPH needed is calculated by eq. (4.47) that can be derived from eqs. (4.8), (4.9), and (4.12). Reducing power can be generated from many different pathways. The synthesis of some nucleotides produces a small amount of NADPH. The part of NADPH produced in the fatty acid synthesis has been deducted from the NADPH requirement for the fatty acid biosynthesis (eq. 4.15), and hence is not included in the production. Mitochondrial transhydrogenase catalyzes the reversible conversion between NADH and NADPH coupled with the transfer of proton across the mitochondrial membrane. The direction of the conversion depends on the mitochondrial proton gradient and the redox ratios. The net production or consumption of NADPH via the transhydrogenase is assumed to be negligible. The above simplifications may cause a significant error in the estimation of NADPH production, but should not cause a significant error in the overall glucose balance. The major NADPH demand is assumed to be met by glucose oxidation in the pentose cycle. The production of one mole of ribose-5-P from glucose is
coupled with the production of two moles of NADPH. The rest of the NADPH requirement is provided by the complete oxidation of glucose to CO$_2$ in the pentose cycle; this yields 12 NADPH per glucose molecule according to eq. (4.13). Hence the total NADPH production is the sum of the above three sources and is given by eq. (4.48).

$$\text{NADPH}_R = 2F_{19} + N_{DNA}(2D_f + D_c)$$  \hspace{1cm} (4.47)

$$\text{NADPH}_p = 2F_{17} + N_{DNA}(D_g + D_a) + 2N_{RNA}(R_g + R_a) + 12F_{20}$$ \hspace{1cm} (4.48)

By assuming that cells produce just enough NADPH for biosynthesis and there is no net accumulation, the amount of glucose completely oxidized for NADPH production ($F_{20}$) can be derived from eqs. (4.47) and (4.48), by balancing the total production with the demand.

$$F_{20} = \frac{2F_{19} + N_{DNA}(2D_f + D_c - D_g - D_a) - 2N_{RNA}(R_g + R_a) - 2F_{17}}{12}$$ \hspace{1cm} (4.49)

According to equations (4.16) and (4.17), the complete oxidation of glucose to carbon dioxide generates 2 ATP, 2 GTP, 8 mitochondrial NADH, 2 cytosolic NADH, and 2 FADH$_2$. It is generally believed that the cytosolic NADH generated from the glycolytic pathway is converted into mitochondrial FADH$_2$ through the glycerol phosphate shuttle. Based on the above analysis, the total amount of ATP per glucose oxidized is determined as:

$$Y_{\text{ATP/gl}} = 4 + 8\eta_v + 4\eta_e$$ \hspace{1cm} (4.50)

The amount of glucose completely oxidized to meet the ATP demand for cell growth is derived from an ATP balance as described by equation (4.51)
\[ \theta_{\text{glc}}^\text{ATP} = \frac{\theta_{\text{ATP}}}{Y_{\text{ATP/glc}}} = F_1 \]  

(4.51)

The overall stoichiometric coefficient of glucose is then the sum of the five stoichiometric requirements for glucose in the syntheses of cell components and energy:

\[ \theta_{\text{glc}} = F_1 + F_{17} + F_{18} + F_{19} + F_{20} \]  

(4.52)

### 4.2.4.5 Stoichiometric Coefficients for Vitamins

Vitamins are essential nutrients for animal cell survival and growth in vitro even though the quantities required are much smaller as compared to those of amino acids and glucose (Eagle, 1955; Lambert and Pirt, 1975). The cell components synthesized from vitamins are mainly enzymes and cell membrane components. The amounts of these components are difficult to measure experimentally due to the large number of species involved. Therefore, the stoichiometric coefficients for vitamins cannot be determined through the same approach as that for amino acids and glucose.

The method used here to estimate the stoichiometric coefficients for vitamins is experimental. Due to the small consumption, the vitamin concentrations in the culture medium are usually very low. Depletion of any essential vitamins can result in cell death, but high concentrations of vitamins show no harm to animal cell growth. Therefore, the most important point here is to ensure that no vitamin depletion occurs during a cultivation process. The above analysis results in the
use of the vitamin yields $Y_{v,i}$ to determine the stoichiometric demands for cell growth. By definition, the stoichiometric coefficients for vitamins are given by:

$$
\theta_{v,i} = 1/Y_{v,i} \quad \text{(Vitamins)}
$$

(4.53)

### 4.3 Stoichiometric Equation for Real System

#### 4.3.1 Metabolic Reaction Network

In a real system, production of by-products such as lactate, ammonia, and non-essential amino acids is unavoidable. These differences result in a much more complicated system and render the system underdefined by the cell composition and product formation. Figure 4.3 depicts the reaction network for glucose and amino acid metabolism for the real system.

The glutamine carbon skeleton enters the TCA cycle as α-ketoglutarate. It has been shown by NMR studies that glutamine carbons exit the cycle via the malate shunt (McKeehan 1986; Sharfstein et al., 1994). There are three possible pathways for the conversion of malate to pyruvate (Mancuso et al., 1994). Mitochondrial malate can be transported across the membrane into cytosol, and converted into pyruvate catalyzed by a NADP⁺-linked cytosolic malic enzyme. It can be converted to pyruvate by a NAD(P)⁺-linked mitochondrial malic isozyme. It is also possible that mitochondrial malate is converted to oxaloacetate and then to phosphoenolpyruvate which is
Figure 4.3 A part of the metabolic reaction network for animal cell growth
transported into the cytosol, and then converted into pyruvate in the glycolytic pathway. The above three pathways are lumped into a single pathway and is shown in Figure 4.3 as the conversion of oxaloacetate to pyruvate since malate is not shown.

Nine new metabolites are introduced into the network shown in Figure 4.3, which provides nine degrees of freedom. Without additional conditions or restrictions, there is no unique solution to the above system. This explains why cell culture performance may differ from batch to batch resulting from small changes in culture environment.

4.3.2 Design Criteria for Process Optimization

The goal for studying the stoichiometric nutritional demand for cell growth is to maximize cell culture performance. It is obviously pointless to solve the above metabolic reaction network using experimentally measured values from a batch culture, because the inefficiency of nutrient utilization is inherent in these values. Our approach to the above problem is to study the conditions that lead to optimized culture performance as described below.

As previously shown, by-product formation needs to be minimized to increase efficiency of nutrient utilization and to increase culture performance. In order to reduce lactate production, glucose concentration needs to be maintained at a low level but sufficient to meet a desired cell growth rate. Because majority of
the ammonia production is derived from glutamine, it is important to understand how ammonia is formed in order to develop a strategy for its reduction.

Figure 4.4 shows the fates of nitrogens in glutamine metabolism. The amide group in glutamine plays an essential role in DNA and RNA synthesis. However, only a small amount of glutamine is consumed for this purpose. The amide group can also be converted into free ammonia through enzymatic hydrolysis via glutaminase. The non-enzymatic degradation of the glutamine amide group is negligible due to the low glutamine level required to reduce ammonia formation. The amino group in glutamine has different fates. It can be hydrolyzed by glutamate dehydrogenase. This reaction produces free ammonia and α-ketoglutarate. The amino group can also be used for the synthesis of non-essential amino acids via aminotransferases. The α-ketoglutarate produced from hydrolysis and aminotransfer reactions enters the TCA cycle for energy production.

Obviously, to reduce ammonia formation, one needs to reduce or eliminate $F_{5,a}$ and $F_{6,a}$. From the material balance of glutamine carbon skeleton, the following equation can be derived from Figure 4.4:

$$F_5^a + F_5^b = F_6^a + F_6^b$$  \hspace{1cm} (4.54)

The total ammonia production from glutamine is:

$$F_6^a + F_6^b = 2F_5^a + F_5^b - F_6^b$$  \hspace{1cm} (4.55)

The material balances of non-essential amino acids yields

$$\theta_{a,i} = U_{a,i} + S_{a,i} \hspace{1cm} \text{(for seven non-essential amino acids)}$$  \hspace{1cm} (4.56)
Figure 4.4 Glutamine metabolism and non-essential amino acid synthesis
and

\[ F^b_3 = \sum_{i=1}^{7} S_{a_i} \quad (4.57) \]

It is clear from equation (4.55) that ammonia released from the amino group is most undesirable due to the coupled ammonia production from the amide group of glutamine. The biosynthesis of non-essential amino acids from glutamine also increases the total ammonia production even though no ammonia is formed directly from the aminotransfer reaction. From equation (4.56), it is obvious that uptake of non-essential amino acids from culture medium reduces ammonia production by alleviating the burden of biosynthesis from glutamine.

On the basis of the above analysis, the following design criteria are employed to ensure minimum lactate and ammonia production: (1) the glutamate hydrolysis can be reduced and hence neglected by maintaining a low glutamine concentration; (2) the non-essential amino acid uptake should be maximized while minimizing the biosynthesis from glutamine; (3) a minimum lactate to glucose ratio can be achieved by maintaining glucose and glutamine at low concentrations during cell growth. The first criterion introduces a restraint to the model. That is the total amount of amino group from glutamine in the biosynthesis should balance with the total amount of non-essential amino acids synthesized. Therefore, with one restraint, seven parameters from the maximum non-essential amino acid uptake, and the minimum lactate to glucose ratio, the degree of freedom for the real system now decreases to zero. A unique solution can now be obtained.
4.3.3 Determination of Stoichiometric Coefficients

In the real system, the stoichiometric coefficients of product, ATP, essential amino acids (except glutamine), and vitamins are the same as in the ideal system. The stoichiometric coefficients for glucose, glutamine, and non-essential amino acids are described below. Because glucose and glutamine metabolism is coupled in the real system, the determination of the reaction network is necessary to obtain the stoichiometric coefficients. The reaction extents of \( F_{17}, F_{18}, F_{19}, \) and \( F_{20} \) in Figure 4.3 are defined by cell composition alone, and hence are the same as in the ideal system.

It is hypothesized that there is no net consumption or accumulation of any intracellular intermediate. The following equations can be derived using material balances of pyruvate and TCA cycle intermediates.

\[
2F_1 + F_{10} = F_2 + F_4 + F_{13} \quad (4.58)
\]

\[
F_{13} = F_3 \quad (4.59)
\]

\[
F_{23} = F_3 + F_5 \quad (4.60)
\]

\[
F_{24} = F_{23} \quad (4.61)
\]

\[
F_{26} = F_{24} + F_{25} \quad (4.62)
\]

\[
F_{26} = F_3 + F_8 + F_{10} \quad (4.63)
\]

where \( F_{25} \) is the amount of fumarate produced in the biosynthesis of nucleotides and can be derived from equations (4.6), (4.7), (4.10), and (4.11).

\[
F_{25} = \theta_{\text{asp}}^{\text{nuc}} \quad (4.64)
\]
A material balance on each non-essential amino acid yields:

\[ F_4 = \theta_{\text{ala}}^p - U_{\text{ala}}^{\max} \]  
\[ F_7 = \theta_{\text{pro}}^p - U_{\text{pro}}^{\max} \]  
\[ F_9 = \theta_{\text{asn}}^p - U_{\text{asn}}^{\max} \]  
\[ F_8 = \theta_{\text{asp}}^p + \theta_{\text{asp}}^{\text{nuc}} + F_9 - U_{\text{asn}}^{\max} \]  
\[ 2F_{11} = \theta_{\text{ser}}^p + \theta_{\text{gly}}^{\text{ser}} + F_{12} - U_{\text{ser}}^{\max} \]  
\[ F_6 = F_5 + \theta_{\text{glu}}^p + \theta_{\text{glu}}^{\text{nuc}} + F_7 - U_{\text{glu}}^{\max} \]

The amount of glutamate converted into \( \alpha \)-ketoglutarate should equal the amount of amino group that is transferred into non-essential amino acids, because no amino group is allowed to be hydrolyzed according to the design criteria. This can be interpreted as:

\[ F_5 = F_4 + F_8 + 2F_{11} \]

Material balances on glucose and glutamine yield:

\[ \theta_{\text{gluc}} = F_1 + F_{11} + F_{17} + F_{18} + F_{19} + F_{20} \]
\[ \theta_{\text{gin}} = \theta_{\text{gin}}^p + \theta_{\text{gin}}^{\text{nuc}} + F_6 \]

The total amount of ATP produced can be expressed as a function of the reaction extents:
\[
\theta_{\text{ATP}} = [2F_1 + F_{24}] + [F_3 + 4F_{11} + F_{13} + F_{23} + F_{26}] \eta_n + [2F_1 - F_2 + F_{24}] \eta_f \tag{4.74}
\]

where \( F_2 \) is determined by the molar ratio of lactate to glucose as shown by equation (4.75); \( F_{10} \) and \( F_3 \) are derived from equations (4.58) through (4.63).

\[
F_2 = \theta_{\text{gl}} Y_{\text{laco/gluc}}^{\text{min}} = (F_1 + F_{11} + F_{17} + F_{18} + F_{19} + F_{20}) Y_{\text{laco/gluc}}^{\text{min}} \tag{4.75}
\]

\[
F_{10} = F_5 + F_{25} - F_8 \tag{4.76}
\]

\[
F_3 = 2F_1 + F_5 + F_{25} - F_2 - F_4 - F_8 \tag{4.77}
\]

Insert equations (4.59), (4.60), (4.61), (4.63), (4.75), (4.76), and (4.77) into equation (4.74) and solve for \( F_1 \):

\[
F_1 = \frac{\theta_{\text{ATP}} \cdot F_5 (2 + 6\eta_n + 2\eta_f) + (4F_{11} + F_{25})\eta_n + (1 + 4\eta_n + \eta_f)(F_{25} - F_4 - F_8)}{[(1 + 4\eta_n + 2\eta_f)(F_{11} + F_{17} + F_{18} + F_{19} + F_{20}) Y_{\text{laco/gluc}}^{\text{min}}] - \frac{4 + (4\eta_n + 2\eta_f)(2 - Y_{\text{laco/gluc}}^{\text{min}} - Y_{\text{laco/gluc}}^{\text{min}})}{4 + (4\eta_n + 2\eta_f)(2 - Y_{\text{laco/gluc}}^{\text{min}} - Y_{\text{laco/gluc}}^{\text{min}})}} \tag{4.78}
\]

The reaction extents have now been expressed as functions of cell composition, energy requirement, maximum NAA uptake, and lactate production. The stoichiometric coefficients for glucose and glutamine are defined by equations (4.72) and (4.73) as functions of reaction extents. The stoichiometric coefficient is defined as the amount of extracellular nutrient needed for cell growth. Therefore, the stoichiometric coefficients for non-essential amino acids are different from those defined in the ideal system because the intracellular
biosynthesis from glutamine needs to be considered. In the real system, the
stoichiometric coefficient should equal the uptake as shown by equation (4.79).

\[ \theta_{\alpha_i} = U_{\alpha_i}^{\text{max}} \quad \text{(for non-essential amino acids)} \]  

(4.79)
5. Medium Development and Process Control

5.1 Initial Medium Design

It has been shown that the formation of ammonia and lactate is affected by the concentrations of glutamine and glucose (Hu et al., 1987; Ljunggren and Haggstrom, 1994; Miller et al., 1989a,b). The goal is to design an optimal nutritional environment where the specific production rates of ammonia and lactate can be considerably decreased. Through this approach, the cells can be maintained viable for an extended period before the by-products become detrimental. As a consequence, a higher product concentration can be anticipated.

It is more important to decrease ammonia formation than to decrease lactate formation due to the higher toxicity of ammonia to cells. The ammonia production from glutamine can be decreased by maintaining a low glutamine concentration during cultivation. This is because the non-enzymatic ammonia formation from glutamine can be reduced since the reaction is first order with respect to glutamine concentration. On the other hand, an extremely low glutamine level can trigger a process called apoptosis or programmed cell death (Mercille and Massie, 1994; Singh et al., 1994) and hence is detrimental to cell growth. Apparently, there is an optimal glutamine concentration in the pursuit of reducing ammonia formation and maintaining a reasonable cell growth rate. Unfortunately, this has not been established in the literature.
Glutamine concentration is usually maintained above 1 mM to prevent starvation due to lack of an optimal control policy. The problem with ammonia and lactate accumulation has never been completely solved. It is therefore necessary to investigate the kinetics for cell growth and by-product formation. The apparent $K_m$ for glutaminase was reported to be in the range of 2 - 8 mM (Goldstein and Schooler, 1967; Katunuma et al., 1973; Quesada et al., 1988). The $K_m$ for glutamine in cell growth was found to be 0.15 mM for hybridoma CRL-1606 (Glacken et al., 1989), 0.53 mM for hybridoma 20-8-4S, and 0.21 mM for human cutaneous T cell lymphoma (HuT-78) (Truskey et al., 1990). Assuming Monod kinetics to be valid and comparing the $K_m$ values, the enzymatic degradation of glutamine can be decreased substantially without reducing the cell growth rate to an undesirable value. For example, if the glutamine concentration is maintained at twice the $K_m$ value for cell growth, the enzymatic degradation of glutamine can be minimized since the $K_m$ for glutaminase is substantially greater. However, it should be noted that maintaining such a low glutamine concentration is difficult and could be catastrophic without a rational feeding strategy.

Similarly, maintaining a low glucose concentration can decrease lactate formation, as can be seen by comparing the $K_m$ values for the glycolytic pathway and for cell growth. The apparent $K_m$ in the glycolytic pathways was reported to be 2 mM for glucose in chick embryo fibroblast cultivation (Fagon and Racker, 1978), whereas the apparent $K_m$ of glucose for cell growth was found to be 0.5 mM for hybridoma cells (AB2-143.2) (Martens et al., 1993). It is hypothesized that decreasing the glucose concentration will reduce the
pyruvate flux and thus limit the formation of lactate without significantly affecting the specific cell growth rate. The $K_m$ values for glutamine and glucose in cell growth kinetics were assumed to be 0.15 mM and 0.75 mM respectively for hybridoma CRL-1606 cells. In order to maintain a reasonable initial growth rate and allow some room for feeding deviations, the concentrations of glutamine and glucose in the initial media were formulated to be 0.2-0.5 mM and 0.5-2.0 mM, respectively.

Three generations of the initial medium (Table 3.1) were developed for hybridoma cultures. The concentrations of inorganic salts in the initial media were similar to those in IMDM to maintain the proper osmolality for cell growth. The vitamin concentrations were also the same as those in conventional media. Very low amino acid concentrations were employed in the first generation of the initial medium, Init-A, based on the assumption that this reduction can decrease ammonia formation from these amino acids via decreased glutamate formation in the mitochondria. The results obtained from the fed-batch cultures (FB1 and FB2) using Init-A for feeding indicate that addition of non-essential amino acids (NAA) leads to low ammonia formation. This experience was incorporated into the design of the second generation of the initial medium, Init-B, in which the concentrations of NAAs were significantly increased. Because of the low initial concentrations of the essential amino acids, nutrient depletion could occur due to small errors in feeding. Hence, the concentrations of amino acids (except glutamine and alanine) in Init-C were elevated to 0.5 mM.
A serum-free initial medium (Init-D, see Table 3.2) was developed for CHO cultures. The pH buffer capacity was significantly improved by using 25 mM Hepes and 10-25 mM MOPS. The concentration of sodium bicarbonate was reduced from 50 mM in Init-A to 35 mM. This was done because of the speculated CO₂ accumulation in the culture medium during cell growth. Components such as fatty acids (Kobayashi et al., 1994), reducing reagents (Brown et al., 1983; Hoekstra, 1975), shear stress protecting reagents (Jordan et al., 1994; Murhammer and Goochee, 1990; Papoutsakis and Michaels, 1993; Tan et al., 1994), and trace metals (Hamilton and Ham, 1977; Brandi et al., 1992) were included to replace the essential roles of animal sera. The addition of Primatone RL, a meat hydrolysate, was found to be essential to maintain optimal cell growth. Insulin was also supplemented to the medium. The requirement for transferrin can be met by using a high concentration of ferric citrate.

5.2 Supplemental Medium Design

The composition of the initial medium was not designed to meet the stoichiometric demands for nutrients needed for high cell densities. Therefore, nutrients required for the synthesis of cell mass and product should be supplied to the cells during a culture. It is hypothesized that a relatively constant nutritional environment is optimal for animal cell growth. The concentrations of glutamine and glucose should be maintained fairly constant but at a prescribed low level to decrease ammonia and lactate formation. Hence, the composition of the supplemental medium needs to be determined according to the
stoichiometric ratios of these nutrients as described in Chapter 4. The information needed to determine the stoichiometric equation is discussed below.

5.2.1 Information for the Determination of Stoichiometric Equation

Four different supplemental media (Table 3.5) were formulated for hybridoma and CHO cultures. Sup-1 was designed using cell composition (Table 5.1) obtained from literature (Darnell et al., 1990; Griffiths and Riley, 1985; Smit et al., 1982). The composition of hybridoma CRL-1606 cells (Table 5.1) was later measured and employed for the design of Sup-II and Sup-III. In the design of Sup-IV, the cellular composition (except the dry cell weight) for CHO cells was assumed to be the same as that of the hybridoma cells. The dry cell weight for CHO cells producing interferon-γ was measured to be 40 mg/10⁸ cells.

The molar percentages of the amino acids in cellular proteins cannot be obtained from the literature. In the design of Sup-I, the average composition of the amino acids (Table 5.2) were determined from 207 random proteins with known amino acid sequences (Klapper, 1977). These parameters (Table 5.2) were later measured from the hybridoma cells (CRL-1606) for the design of Sup-II, Sup-III, and Sup-IV. The molar percentages of the amino acids in the product were not measured, but assumed to be the same as those of the cellular proteins. Because product formation is usually a small percentage of the cellular proteins, this simplification should not cause a significant error.
Table 5.1 Cell composition employed to determine the stoichiometric equation for the design of the supplemental media for hybridoma and CHO cell cultures.

<table>
<thead>
<tr>
<th>Cell Composition</th>
<th>Sup-I</th>
<th>Sup-II &amp; Sup-III</th>
<th>Sup-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry cell weight (W)</td>
<td>36 mg/10^6 cells</td>
<td>25 mg/10^6 cells</td>
<td>40 mg/10^6 cells</td>
</tr>
<tr>
<td>Dry weight percentage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins (Z_p)</td>
<td>37.5%</td>
<td>72.9%</td>
<td>72.9%</td>
</tr>
<tr>
<td>DNA (Z_o)</td>
<td>2.5%</td>
<td>1.4%</td>
<td>1.4%</td>
</tr>
<tr>
<td>RNA (Z_R)</td>
<td>6.9%</td>
<td>3.8%</td>
<td>3.8%</td>
</tr>
<tr>
<td>Lipids (Z_L)</td>
<td>20.0%</td>
<td>13.5%</td>
<td>13.5%</td>
</tr>
<tr>
<td>Carbohydrates (Z_C)</td>
<td>25.0%</td>
<td>3.5%</td>
<td>3.5%</td>
</tr>
</tbody>
</table>

Table 5.2 The molar percentages of amino acids in cellular proteins (O^{sp}) used to determine the stoichiometric equation for the design of the supplemental media for hybridoma and CHO cell cultures.

<table>
<thead>
<tr>
<th></th>
<th>ala</th>
<th>arg</th>
<th>asn</th>
<th>asp</th>
<th>cys</th>
<th>glu</th>
<th>gln</th>
<th>gly</th>
<th>his</th>
<th>ile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sup-I</td>
<td>9.0</td>
<td>4.7</td>
<td>4.4</td>
<td>5.5</td>
<td>2.8</td>
<td>6.2</td>
<td>3.9</td>
<td>7.5</td>
<td>2.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Sup-II, -III, -IV</td>
<td>8.3</td>
<td>5.9</td>
<td>4.4</td>
<td>4.7</td>
<td>2.8</td>
<td>6.2</td>
<td>5.0</td>
<td>8.5</td>
<td>2.2</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>leu</td>
<td>lys</td>
<td>met</td>
<td>phe</td>
<td>pro</td>
<td>ser</td>
<td>thr</td>
<td>trp</td>
<td>tyr</td>
<td>val</td>
</tr>
<tr>
<td>Sup-I</td>
<td>7.5</td>
<td>7.0</td>
<td>1.7</td>
<td>3.5</td>
<td>4.6</td>
<td>7.1</td>
<td>6.0</td>
<td>1.1</td>
<td>3.5</td>
<td>6.9</td>
</tr>
<tr>
<td>Sup-II, -III, -IV</td>
<td>8.1</td>
<td>6.8</td>
<td>2.2</td>
<td>3.2</td>
<td>5.3</td>
<td>6.9</td>
<td>5.7</td>
<td>1.1</td>
<td>2.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>
The base composition of cellular DNA and RNA was not measured and unavailable in the literature. In general, each base is approximately 25% of the total bases in DNA and RNA (Campbell, 1990). Furthermore, the amino acid requirements for DNA and RNA synthesis are not sensitive to the base composition (Kelley, 1972). Therefore, the molar percentage of each base was assumed to be 25% of the total.

The cellular yields on vitamins (except for riboflavin and vitamin B_{12}) were obtained from the literature (Lambert and Pirt, 1975) which were determined from a culture of human diploid cells (MRC-5). The yields of riboflavin and vitamin B_{12} were estimated from a batch culture of hybridoma CRL-1606 cells by assuming complete consumption of these two vitamins at the end of the cultivation. The yields (Y_{kl}, \mu mole/10^8 cells) used in the determination of the vitamin coefficients are: biotin, 0.778; choline, 38.3; folic acid, 1.4; inositol, 20.4; nicotinamide, 5.57; pyridoxal, 7.66; riboflavin, 0.5; thiamine, 1.27; pantothenate, 1.6; and vitamin B_{12}, 0.1.

The specific cell growth rate, was assumed to be 0.035 h\(^{-1}\) (Sup-I and Sup-II), 0.03 h\(^{-1}\) (Sup-III), and 0.02 h\(^{-1}\) (Sup-IV). The specific product synthesis rate of hybridoma cells was assumed to be constant. In a fed-batch culture of hybridoma CRL-1606, the specific antibody synthesis rate was reported to be 0.6 \times 10^{-9} \text{ mg/cell-h} (Lindell, 1992), which was used in the design of Sup-I and Sup-II. A specific product synthesis rate of 0.9 \times 10^{-9} \text{ mg/cell-h} was measured from the fed-batch culture using Sup-II for feeding (FB3) and was employed in the design for Sup-III. The specific product synthesis rate for CHO cells was
estimated from batch cultures as $0.1 \times 10^{-9}$ mg/cell-h in the design of Sup-IV. The specific ATP consumption rate was taken from a continuous culture of hybridoma cells (AB2-143.2) (Miller et al., 1989b) to be:

$$q_{\text{ATP}} = \mu / [7.48 \times 10^{7}] + 6 \times 10^{-10} \text{ mmole ATP/cell-h} \quad (5.1)$$

This value was employed in the design of all four supplemental media. The P/O ratio was assumed to be 3 in the design of Sup-I, II, and III; and 2 (see 7.4.1) in the design of Sup-IV. The molar ratio of lactate to glucose was assumed to be 0.5.

In the development of Sup-I, the specific uptake of NAAs was assumed to be zero. It was later hypothesized that the uptake of NAAs from culture medium could reduce the glutamine requirement and hence reduce ammonia production (see Chapter 4). In the design of Sup-II, the maximum uptake of NAAs (Table 5.3) was calculated from fed-batch cultures performed with Sup-I (FB1 and FB2). The measurements from FB3 that was fed with Sup-II was employed to determine the maximum uptake of NAAs for the design of Sup-III. In the design of Sup-IV, the results from the hybridoma fed-batch culture (FB3) were used to calculate the uptake of NAAs for CHO cells by simply multiplying the dry cell weight ratio of CHO cells to hybridoma cells.

### 5.2.2 Supplemental Medium Composition

The supplemental medium needs to be designed in such a way that the nutrient composition is stoichiometrically balanced with the demands for cell growth. An
Table 5.3 The maximum uptake of NAAs (mmole/10⁹ cells) employed to determine the stoichiometric equation for the design of the supplemental media for hybridoma and CHO cell cultures.

<table>
<thead>
<tr>
<th></th>
<th>Sup-II</th>
<th>Sup-III</th>
<th>Sup-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>-0.17</td>
<td>-0.17</td>
<td>-0.3</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.046</td>
<td>0.094</td>
<td>0.136</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.13</td>
<td>0.119</td>
<td>0.206</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.076</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.082</td>
<td>0.047</td>
<td>-0.047</td>
</tr>
<tr>
<td>Proline</td>
<td>0.0</td>
<td>0.0</td>
<td>-0.05</td>
</tr>
<tr>
<td>Serine</td>
<td>0.048</td>
<td>0.092</td>
<td>0.272</td>
</tr>
</tbody>
</table>
optimal feeding strategy is to maintain the nutritional environment reasonably constant during cell growth. This requires that the amount of nutrients provided to the cells be equal to the consumption. Such a feed-on-demand strategy can be expressed as:

\[ V_f C_i = \theta_i \Delta N_i \]  \hspace{1cm} (5.2)

Define \( C_i \) as the total nutrient concentration in the supplemental medium and \( \beta \) as the total stoichiometric coefficient:

\[ \beta = \sum_{i=1}^{\theta_i} \]  \hspace{1cm} (5.3)

\[ C_t = \sum_{i=1}^{\theta_i} C_i \]  \hspace{1cm} (5.4)

Insert equations (5.2) and (5.3) into equation (5.4):

\[ C_i = \frac{\beta \Delta N_i}{V_f} \]  \hspace{1cm} (5.5)

and combine equations (5.2) and (5.5) to solve for \( C_i \):

\[ C_i = \frac{\theta_i}{\beta} C_t \]  \hspace{1cm} (5.6)

The total concentration of glucose, amino acids, and vitamins in the supplemental medium, \( C_t \), is related to the osmolality of the supplemental medium. When the concentrations of glucose and glutamine are maintained low, the formation of ammonia and lactate should decrease significantly. Hence, the effect of by-product formation on osmolality increase will be reduced significantly. The consumption of low molecular weight species will decrease the medium osmolality during cell growth. In view of this behavior, the osmolality in the medium was assumed to decrease during cell growth. Hence, it is rationalized that the osmolality of the supplemental medium should be
greater than the osmolality in the reactor. Thus the total solute concentrations, which is approximately equal to the osmolality of the supplemental medium, was set to be 400 mM in Sup-I based upon the above analysis. The total nutrient concentration in Sup-II was originally designed to be 400 mM. However, difficulties were encountered in dissolving some of the amino acids, hence the total nutrient concentration was reduced to 250 mM. The osmolality of the culture medium decreased during the fed-batch culture using Sup-II for feeding (FB3). This suggests that the total nutrient concentration in the supplemental medium should be increased to maintain a constant osmolality in the bioreactor. The total nutrient concentrations in Sup-III and Sup-IV were increased to 600 mM after the solubility problems were solved through elevating the pH of the supplemental media.

The determination of the stoichiometric coefficients for glucose, amino acids, and vitamins has been described above. The concentrations of these nutrients in the supplemental media can then be calculated from equation (5.6).

5.3 Process Control

In the T-flask cultures, no process control was implemented. The execution of the feeding was manual and periodical. Samples were taken every 12 - 24 hours. Off-line measured cell densities were employed to project the cell growth in the time interval between the two subsequent samples. The volume of the supplemental medium to be fed (about an hour after sampling) can be calculated using equation (5.7) which is derived from equation (5.5):
\[ V_I = \frac{\beta \Delta N_I}{C_I} \]  \hspace{1cm} (5.7)

The net increase of the total cell number in the bioreactor during a specific time interval can be estimated from the cell growth kinetics as:

\[ \Delta N_I = \frac{\mu N_{V,n}}{\mu - \alpha} \left[ e^{(\mu - \alpha) \Delta t} - 1 \right] \]  \hspace{1cm} (5.8)

where the specific growth and death rates were calculated using cell densities from two adjacent samples according to equations (3.17) and (3.18).

In the bioreactor cultures, the dissolved oxygen (DO) and pH were controlled by adjusting the inlet gas composition. Values of DO and pH measured with Ingold probes were inputted into a real time controller computer. A PID control module provided by the design program was employed to adjust the inlet oxygen and carbon dioxide percentages for the control of DO and pH at their set-points of 60 ± 1% and 7.2 ± 0.005, respectively.

The control of the nutritional environment was achieved through the feeding of a supplemental medium. The control criterion was to feed the supplemental medium according to the actual consumption rates of nutrients. The volume of the supplemental medium required to compensate for the consumption of nutrients in the bioreactor was estimated from equation (5.9). The net increase of the total cell number was calculated according to equation (5.10).

\[ V_R = \frac{\beta \Delta N_I}{C_I} \]  \hspace{1cm} (5.9)

\[ \Delta N_I = N_I - V_0 X_{t,0} \]  \hspace{1cm} (5.10)
The total cell number in the reactor at culture time \( t \) was calculated from the off-line cell density measurements according to equation (5.11).

\[
N_t = N_t^n + \frac{\mu N_{v,n}}{\mu - \alpha} \left[ e^{(\mu - \alpha)(t - t_n)} - 1 \right]
\]

(5.11)

Where \( N_t^n \) is the total cell number in bioreactor at the time when the \( n \)th sample was taken. The initial total cell number, \( N_t^1 \), was calculated from the initial culture volume and the total cell density measured from the first sample taken at time zero. The total cell number at the time when each subsequent sample was taken (\( n > 1 \)), was calculated from equation (5.11) using \( N_t^{n-1} \), and then assigned to \( N_t^n \). Estimations of \( N_{v,n} \) and specific cell death rate were based on cell density measurements from the (\( n-1 \)) and \( n \)th samples using equations (3.19) and (3.17), respectively. A constant specific growth rate of 0.03 h\(^{-1}\) was employed for the total cell number calculation throughout the cultivation. However, the total cell number in the bioreactor \( (N_t^n) \) was not adjusted according to the measurement from samples. This is because the maximum error in cell enumeration could be as great as 10%. If a total cell density with a 10% error were inputted into the computer, and the total cell number were adjusted based on this inaccurate measurement, starvation or overfeeding would have resulted. Similarly, calculation of the specific growth rate using two sequential measurements could be significantly different from the real value. The rationale of using a constant growth rate is described in more detail below.

If we assume cell growth follows the Monod growth model, the growth rate should be a function of nutrient concentrations. When the concentrations of all essential nutrients are much greater than their \( K_m \) values, a maximum specific
growth rate should result. When one or several essential nutrients are below their \( K_m \) values, the specific growth rate would be approximately a linear function of the concentrations of the limiting nutrients. The concentrations of glucose and glutamine in the initial media were designed to be very low in an effort to reduce toxic ammonia and lactate formation. Under these conditions, the specific growth rate is therefore governed by the glucose and glutamine concentrations. When the real growth rate in the reactor is greater than the growth rate used to calculate the feeding rate, glucose and glutamine concentrations will decrease. This will then return the actual growth rate back to the value used for feeding. When the actual growth rate is lower, the opposite will occur. Therefore, stable cell growth during the entire culture process can be achieved if the glutamine and glucose concentrations are controlled at low levels.

The feeding of the supplemental media was achieved through the control of a feed pump in the following fashion. Samples were taken every 12 hours during the process. Total and viable cell densities were determined immediately, and were inputted into a real time computer along with the culture time when the sample was taken. The measured viable cell density and calculated specific death rate were then used to project the total number of cells produced at time \( t \) according to equations (5.10) and (5.11). The required volume of the supplemental medium \( (V_R) \) was then determined using equation (5.9). Signals from a balance for measuring the weight of the supplemental medium were transferred through a BCD interface to a real time computer. The total volume of the supplemental medium already fed to the bioreactor \( (V_p) \) was then
determined by the weight change and the supplemental medium density (1.05 g/ml). When the required volume exceeded the total volume already fed, the feed pump was turned on. The accuracy of the balance is 0.1 gram. Hence, after 0.1 gram of the supplemental medium had been fed (in about 10 seconds), the total volume fed to the reactor would then exceed the required volume, and meeting this condition would turn the feed pump off. As the viable cell density increased with culture time, the feed pump was turned on/off more and more frequently.
6. Hybridoma Cultures

6.1 Hybridoma Cell Growth

6.1.1 First Generation Study

Fed-batch cultures were carried out with T-flasks to examine the cell growth with the feeding of Sup-I (Table 3.5). The culture was initiated with the medium Init-A which contains very low concentrations of glucose and amino acids. The feeding of the supplemental medium was executed every 12 hours, according to the devised feeding strategy (see Chapter 5). Samples were taken every 12-24 hours, and the cell density measured. No improvement over conventional batch cultures was attained with respect to the maximum viable cell density in six days.

There are two hypotheses which could explain the above behavior: either glutamine was insufficient due to its consumption for the synthesis of non-essential amino acids, or glucose was insufficient due to lactate formation. Both factors were neglected in the stoichiometric analysis of the ideal system. Two parallel experiments were then performed to examine these two hypotheses. One was fed with Sup-I plus extra glucose. The second one was fed with Sup-I plus extra glutamine. The amounts of extra glucose and glutamine were determined based on the cell density. This was designated "extra" because theoretically the feeding amount of Sup-I should have satisfied the stoichiometric demands for cell growth.
The total cell densities obtained from the above experiments and conventional batch cultivation are shown in Figure 6.1. The total cell densities in the experiments fed with Sup-I alone and with extra glucose were nearly identical. Both experiments showed lower growth rate as well as lower maximum total cell density as compared to those of the batch culture. Hence, both experiments were kinetically and stoichiometrically limited by nutrients other than glucose. On the other hand, when Sup-I plus extra glutamine was fed, the total cell density reached $7.5 \times 10^6$ cells/ml at 150 hours, twice the maximum cell density in the batch culture. This behavior strongly suggests that glutamine in the supplemental medium Sup-I was insufficient, probably due to neglecting the consumption of glutamine for the synthesis of non-essential amino acids (NAA). It was also hypothesized that the stoichiometric demands for glutamine and NAAs are interrelated and need to be considered to improve the design of the supplemental medium.

6.1.2 Second Generation Study

Results obtained from the first generation study suggest that the concentrations of glutamine and NAAs need to be assessed and revised. Specifically, the glutamine concentration needs to be increased while the concentrations of NAAs need to be decreased. The requirement of glutamine for the synthesis of NAAs was analyzed in order to define the concentrations of glutamine NAAs in the supplemental medium.
Figure 6.1 Comparison of hybridoma cell growth in T-flask batch and fed-batch cultures fed with Sup-I plus extra glutamine or glucose in the first generation study
From the literature as well as from previous experience, the production of alanine is known to exceed the stoichiometric demand for cell growth (Duval et al., 1991; Ljunggren and Haggstrom, 1992; Miller et al., 1989). Also, the synthesis of proline and asparagine can be assumed to be sufficient for cell growth due to the small amounts required. Hence, alanine, proline, and asparagine were removed from the supplemental medium Sup-I. The concentrations of serine, glycine, and aspartate were also decreased since they can be synthesized from glutamine. However, 4 mM serine, 5 mM glycine, and 5 mM aspartate were formulated into the new supplemental medium (denoted Sup-IA hereafter) considering their roles in the synthesis of nucleotides. Because most NAAs are derived directly from glutamate, the concentration of glutamate was increased from 0 mM in Sup-I to 10 mM in Sup-IA. This was done based on the assumption that extracellular glutamate can partially substitute for the glutamine in the synthesis of other NAAs. The sum of glutamine and NAAs in the Sup-I was 81.9 mM. The sum of the concentrations of NAAs in Sup-IA was 24 mM. By assuming that the same total amount of glutamine and NAAs is required to satisfy the stoichiometric demands, the glutamine concentration was thus calculated to be 57.9 mM. The concentrations of the other components are identical in Sup-I and Sup-IA.

Figure 6.2 shows the cell growth using consecutive feeding of Sup-I plus extra glutamine for the first 160 hours and then followed by the feeding of Sup-IA. This experiment will be denoted as FB1. At 160 hours, when the cell density was already quite high, cells were then divided into two new T-175 flasks in
Figure 6.2 Comparison of hybridoma cell growth in T-flask batch and fed-batch cultures using consecutive feeding.
order to eliminate the possibility of oxygen limitation. The maximum total and viable cell densities reached $2.1 \times 10^7$ cells/ml and $6.1 \times 10^6$ cells/ml respectively. The viable cell density reached a maximum level and stayed constant for more than 100 hours while the total cell density increased over the entire culture span. The inability to continuously increase the viable cell density was probably due to an oxygen limitation resulting from the poor mass transfer in the T-flasks. This is supported by the fact that splitting the culture into two T-flasks at 160 hours increased the viable cell density from $3 \times 10^6$ cells/ml to $6 \times 10^6$ cells/ml, with the total cell density increasing continuously until the end of the experiment. The culture span was successfully prolonged to 400 hours. In comparison, total cell density in conventional batch cultivation was only $3.7 \times 10^6$ cells/ml, and the viable cell density was only $2.8 \times 10^6$ cells/ml, with a culture span of 150 hours.

In order to confirm that Sup-IA alone can support growth to a higher cell density, a second experiment, denoted as FB2, was conducted with the feeding of Sup-IA for the entire cultivation. The cell densities are shown in Figure 6.3. Both the viable and total cell densities increased continuously until 320 hours when the viable cell density decreased dramatically. The overall viability was increased compared to the previous experiment (Figure 6.2) although the total cell density was slightly lower. These results confirm that the composition of Sup-IA was improved.
Figure 6.3 Hybridoma cell growth in a T-flask fed-batch culture fed with an improved supplemental medium (Sup-IA).
6.1.3 Bioreactor Cultures

The integrated approach for medium design and nutritional control was further verified under controlled conditions in a 2-liter bioreactor. Two fed-batch and one batch cultures were conducted. Dissolved oxygen and pH were controlled by a real time computer. Automatic and continuous feeding of supplemental media (Sup-II and Sup-III) was employed in the two fed-batch cultures. The one fed with Sup-II will be denoted hereafter as FB3, the batch as B4, and the one fed with Sup-III as FB5. Figure 6.4 shows the cell densities from cultures FB3 and B4. In FB3, the specific growth rate was maintained at a low value through the control of nutrient concentrations at low levels. This was preferred because it reduces byproduct formation. Viable cell density reached a maximum of $6.3 \times 10^6$ cell/ml, and then remained constant for more than 100 hours while total cell density was increasing monotonously until the end of the culture. Although it was expected that the automatic feeding and process control would improve the culture performance over the fed-batch cultures in T-flasks, no appreciable improvement was achieved with respect to the maximum viable cell density. Massive cell death was observed at the late stage of all three fed-batch cultures (FB1, FB2, and FB3). Since the dissolved oxygen and pH were well controlled, oxygen transfer or pH change was not the limiting factor for the cell death.

In the fed-batch culture FB5, the viable cell density (Figure 6.5) increased exponentially to about the same value as the previous experiments at 104 h, but
Figure 6.4 Comparison of hybridoma cell growth in bioreactor batch and fed-batch cultures fed with a stoichiometrically designed supplemental medium (Sup-II).
Figure 6.5 Hybridoma cell growth in a bioreactor fed-batch culture using automatic feeding of a stoichiometrically designed supplemental medium (Sup-III).
remained at that value for 24 hours. This was followed by a decrease to $3.67 \times 10^5$ cells/ml in the subsequent 24 hours, due to possible starvation for about 8 hours caused by an accidental fault in the control system at 128 h (hardware problem). The viable cell density then increased to $5.36 \times 10^6$ cells/ml after the resumption of supplemental medium feeding. To explore the possibility of the limitation by growth hormone and trace metals, undialysed fetal bovine serum and trace metals (Fe$^{2+}$, SeO$_3^{2-}$, Li$^+$, Zn$^{2+}$, Cu$^{2+}$, and PO$_4^{3-}$), were manually added to the bioreactor after the viable cell density reached a plateau. As shown in Figure 6.5, the viable cell density increased to a maximum value of $17 \times 10^6$ cell/ml. This represents an important breakthrough in the viable cell density.

The significant increases in the maximum viable cell density and the culture span in FB5 were mainly due to the feeding of undialysed serum and trace metals along with the new feeding strategy (see Chapter 5). Comparison with FB3 and careful observation of the responses from cells to the addition of serum and trace salts show that these nutrients are insufficient in the initial medium to reach a high cell density. A better understanding of the stoichiometric demands for these nutrients is needed in order to include them into the supplemental medium for automatic feeding.

The total cell density was almost the same as the viable cell density in the first 100 hours of FB5, indicating that specific death rate was very low during this period. It then increased continuously in the next 96 hours, while the viable cell density was decreasing. Hence, the specific death rate was greater than the specific growth rate. Due to the increase of the viable cell density, the total cell
density increased to $4.8 \times 10^7$ cells/ml at 453 h. After this time, the total cell density remained essentially constant, indicating possible cell lysis. Dry cell weight per unit volume were found to be 14.7 and 12.3 g/L from samples taken at 453 and 549 hours, respectively. These results confirmed that cell lysis occurred at the late stage. The final cell density (dry cell weight per unit volume) is comparable to that in microbial fermentation, considering the fact that no cell entrapment device was employed. On the other hand, the addition of serum and trace metals failed to prevent cell death completely, indicating that there may be other unidentified limiting factors in the system.

6.2 Control of the Nutritional Environment

6.2.1 Glucose and Glutamine Control

The control of the nutritional environment is vital for an optimal cell growth. This is because by-product formation is affected by glucose and glutamine concentrations in the bioreactor. In addition, maintaining a constant nutritional environment can avoid nutrient depletion and/or accumulation. Since the supplemental media employed for feeding in the fed-batch cultures were formulated according to the stoichiometric equation governing cell growth. Ideally, the feed-on-demand strategy developed for feeding can control the entire nutritional environment even though only one parameter, the feeding rate, is available for control.

In the fed-batch cultures with T-flasks (FB1 and FB2), the glucose concentration (Figure 6.6) was not well controlled, especially in FB1 where about 35 mM
Figure 6.6 Residual concentration of glucose in T-flask fed-batch cultures (FB1 and FB2) using manual feeding.
glucose accumulated in the culture medium at the end. This was partially because the percentages of carbohydrates and lipids in the cell composition was overestimated in the design of Sup-I. Another reason was that an underestimated cell death rate resulted in an overestimation of the cell growth which caused over feeding. The control of glucose in the first 200 hours of FB2 was quite well considering the deviation in cell composition. At the end of FB2, 20 mM of glucose also accumulated in the medium. On the other hand, the glutamine concentration was controlled quite well in both FB1 and FB2. Small perturbation in glutamine control was observed in FB1 probably due to errors in the estimation of the extra glutamine addition. Glutamine was maintained below 0.5 mM during the entire culture course of FB2. Because slight accumulation of other nutrients in the medium was observed, it was suspected that glutamine was one of the limiting nutrients and that its concentration in Sup-IA was underestimated. It was assumed that the spontaneous degradation of glutamine was negligible at a low glutamine concentration and hence was not considered in the design of the supplemental medium. This assumption is justified by the results from FB2. The amount of glutamine undergone spontaneous degradation was estimated to be 0.18 mM using a residual glutamine concentration of 0.1 mM and a total culture span of 360 hours, with a first order degradation constant of 0.0048 h⁻¹. This estimated value (0.18 mM) is only 1.5% of the total 12 mM glutamine consumed.

The control of glucose and glutamine (Figures 6.7 and 6.8) was significantly improved in FB3 through the automatic feeding of an improved supplemental medium (Sup-II) which was formulated according to the experimentally
Figure 6.7 Residual concentrations of glutamine in fed-batch cultures (FB1 and FB2) using manual feeding.
Figure 6.8 Comparison of the residual concentrations of glucose and glutamine in bioreactor batch (B4) and fed-batch (FB3) cultures.
measured cell composition. Unlike the batch culture (B4) where glucose and glutamine were depleted at the end, both glucose and glutamine were maintained at low and constant concentrations over the 350 hour cultivation in FB3. In FB5 where the culture span was increased to 550 hours, the glucose concentration (Figure 6.9) was controlled reasonably well in the first 300 hours. Perturbation in glucose control was observed in the last 200 hours, partially due to the malfunction of the feeding pump over a short period of time. The underestimation of the cell death rate in the stationary phase of cell growth also attributed to the overfeeding. Considering the high viable cell density which resulted in a considerable requirement for nutrients, the glucose control was quite satisfactory in FB5. Similarly, glutamine was controlled at very low level in the first 300 hours (Figure 6.9). Slight increase in glutamine concentration was observed in the last 200 hours but it never exceeded 2 mM. It should be emphasized that the control of glucose and glutamine in the exponential growth phase is much more important than in the stationary phase due to the associated by-product formation in the growth phase (see section 6.3).

### 6.2.2 Control of Essential Amino Acids

The concentrations of essential amino acids in FB1 are shown in Figure 6.10. In the first 120 hours, all essential amino acids except methionine increased with time probably due to the unbalanced nutrient composition in the Sup-I. However, no further increases were observed in the last 200 hours when an improved supplemental medium (Sup-IA) was formulated and employed for
Figure 6.9 Residual concentrations of glucose and glutamine in a bioreactor fed-batch culture using automatic feeding of a stoichiometrically designed supplemental medium (Sup-III).
Figure 6.10 Residual concentrations of essential amino acids in a T-flask fed-batch culture (FB1) using manual feeding.
feeding. In fact, methionine and threonine concentrations decreased in this period of the culture, indicating that the requirement for these two nutrients were underestimated due to errors in the cell composition data used in the stoichiometric analysis. Except arginine, all essential amino acids were controlled below 1.0 mM during the entire culture process. Hence, the accumulation of these amino acids in the culture medium to the osmolality increase was insignificant.

In FB2, the control of the essential amino acids was improved due to the improvement in the supplemental medium (Sup-IA) composition and less of an error in the estimation of the cell death rate (Figure 6.11). Only slight increases in essential amino acids were observed over the 350 hours of cultivation. In fact, the concentrations of methionine, threonine, lysine, isoleucine, and leucine were virtually constant. No amino acids accumulated up to 0.5 mM. Considering the manual feeding and the long culture course, the control of the essential amino acids was satisfactory.

The concentration profiles of essential amino acids in the bioreactor fed-batch culture (FB3) are shown in Figure 6.12. All essential amino acids were controlled at virtually constant levels over the entire culture period of 350 hours. This represents a significant improvement in the control of the nutritional environment. This was partially attributed to the optimized supplemental medium (Sup-II) and the automatic continuous feeding. Small perturbations in the essential amino acid concentrations were observed during the cultivation,
Figure 6.11 Residual concentrations of essential amino acids in a T-flask fed-batch culture (FB2) using manual feeding
Figure 6.12 Residual concentrations of essential amino acids in a bioreactor fed-batch culture (FB3) using automatic feeding of a stoichiometrically designed supplemental medium.
probably caused by the errors in the cell enumeration which was employed to determine the feeding of the supplemental medium. In the exponential growth phase, all amino acids were controlled in the range of 0.15 to 0.5 mM. These results combining with the control of glucose and glutamine prove that the control of the feeding rate according to the feed-on-demand policy allows the control of the entire nutritional environment in fed-batch cell cultivation. It is also quite conclusive that the stoichiometric tools developed for the quantification of the nutritional requirements for animal cell growth are applicable to formulate a stoichiometrically balanced supplemental medium for feeding and control.

6.3 By-product Formation

6.3.1 Lactate

The production of lactate is shown in Figure 6.13. Because of the high glucose concentration in FB1 (Figure 6.6), lactate production was high. Although the profile of the lactate concentration in the first 100 hours of FB1 was almost the same as that of the batch, the specific lactate production rate was lower in FB1 due to the difference in the viable cell density. In FB2, lactate production was further reduced as a result of the improved glucose and glutamine control. Although dramatic reduction was achieved in the T-flask experiments, lactate accumulation (50 mM in FB1 and 30 mM in FB2) was still problematic. No base was employed to neutralize lactate. However, the lactate production itself can lead to osmolality increase (not measured in FB1 and FB2). It also reduced the culture pH and may negatively influence cell growth since no pH control
Figure 6.13 Production of lactate in batch and Fed-batch cultures of hybridoma cells.
was conducted in these cultures. It should also be noted that the instantaneous increase in glucose concentration caused by the periodic feeding was not measured and hence not shown in Figure 6.6. The actual residual glucose concentration should be high than what is shown in the figure because of the periodic and manual feeding.

In the bioreactor cultures (FB3 and FB5), where automatic feeding was employed, the lactate production was reduced to its minimum. Less than 25 mM lactate was produced in FB5 and less than 12 mM in FB3. As compared to the toxic level of greater than 50 mM for the hybridoma cell line employed in these cultures, the problem of lactate accumulation was successfully solved through the design of a stoichiometrically balanced supplemental medium and the implementation of the feed-on-demand strategy. The automatic feeding prevent instantaneous increases in nutrient concentrations in the bioreactor. This allows a more stable control of the nutritional environment (Figure 6.8).

It is very interesting that majority of the lactate was generated in the growth phase. In fact, net consumption of lactate was observed in the stationary phase in FB1, FB3, and FB5. Similar behavior was reported by Luan (1987). It should be noted that the consumption of lactate was not a result of glucose limitation. In FB1, glucose concentration was very high in the last 200 hours of the culture. Sufficient glucose was also available in the culture vessel in FB3 and FB5 when lactate was assimilated into cells. This phenomenon needs further investigation which may reveal the changes of intracellular metabolic pattern that could cause the massive cell death in the stationary phase. It can also be derived that
the control of the nutritional environment is more critical in the exponential growth phase than in the stationary phase.

The evaluation of the control of the nutritional environment should not be based on the net production of lactate. The specific lactate production rate, defined as the amount of lactate produced per cell per unit time, is a more reasonable parameter. As an example, Figure 6.14 shows the comparison of the specific lactate production rate between the batch (B4) and fed-batch (FB5). The decrease in the specific lactate production rate with time in both B4 and FB5 cannot be explained simply by the residual glucose and glutamine concentrations or cell growth rate. Obviously, the control of glucose concentration at a low level was rewarded by a significantly reduced lactate production rate in FB5.

6.3.2 Ammonia

The ammonia profiles in batch and fed-batch cultures are shown in Figure 6.15. Similar to lactate production, majority of the ammonia was produced in the growth phase. However, no significant net consumption of ammonia was observed in all cultures. Comparison of FB1 and FB2 shows that provisions of NAAs in the supplemental medium reduced ammonia production as expected. Accumulation of ammonia is still problematic in the fed-batch cultures since the final ammonia concentrations in all fed-batch cultures exceeded 5 mM. The
Figure 6. Specific lactate production rate in bioreactor batch (B4) and fed-batch (FB5) cultures of hybridoma cells.
Figure 6.15 Production of ammonia in batch and fed-batch cultures of hybridoma cells.
specific production rate shows a dramatic decrease in FB5 as compared to B4 (Figure 6.16). The specific production rate of ammonia decreased with time in both batch and fed-batch. No satisfactory explanation is available for this behavior. The reduction in the ammonia production rate in FB5 indicates that the strategies (glutamine control and NAA addition) employed to minimize ammonia formation were quite successful.

6.3.3 Non-essential Amino Acids

The profiles of non-essential amino acids from the two T-flask cultures are shown in Figures 6.17 (FB1) and 6.18 (FB2) respectively. Alanine in both experiments increased continuously as expected. The final concentration of alanine reached 8.5 mM in FB1 when only 2.8 mM was added. Therefore, there was a net production of 5.7 mM of alanine from glutamine and pyruvate. A significant amount of other NAAs also accumulated in the culture medium. This was probably caused by neglecting the biosynthesis from glutamine since extra glutamine was added during the first 160 hours of FB1. In FB2, the final alanine concentration reached 3.3 mM which was totally synthesized from glutamine since no alanine was fed to the cells. These results confirm the assumption that the synthesis of alanine exceeds the stoichiometric demand. Hence, no alanine needs to be included in the supplemental medium for this cell line. Glutamate accumulated to 2 mM in the end of the culture, indicating that the concentration of glutamate in the Sup-IA was too high. It also suggests that extracellular glutamate may not be able to replace the role of glutamine-derived-glutamate in
Figure 6.16 Specific ammonia production rate in batch (B4) and fed-batch (FB5) cultures of hybridoma cells.
Figure 6.17 Concentrations of non-essential amino acids in a T-flask fed-batch culture (FB1) of hybridoma cells.
Figure 6.18 Concentrations of non-essential amino acids in a T-flask fed-batch culture (FB2) of hybridoma cells.
the biosynthesis of other NAAs. In FB2, glycine, aspartate, asparagine, and serine were all maintained below 0.5 mM, indicating significant improvement as compared to FB1.

The concentrations of NAAs in FB3 are shown in Figure 6.19. Both alanine and glutamate accumulated to over 4 mM at the end of the culture. Since no alanine was included in the feed medium (Sup-II), the accumulation of alanine and glutamate was contributed by both biosynthesis and feeding. Similar to FB2, no significant accumulation of glycine, aspartate, and asparagine was observed. Serine concentration decreased with time in the first 160 hours, but then increased to about 1.4 mM at the end. The shift of serine concentration profile coincides with the decrease in lactate concentration (Figure 6.13). Since the production of serine and lactate is related to glucose metabolism, the above phenomenon suggests that a change in glucose metabolism pattern might have occurred at 160 hours of the culture. Combining the results obtained from FB2 and FB3, one can draw the conclusion that the feeding strategy and the stoichiometric medium design can be employed to control not only the concentrations of glucose and essential amino acids but also some of the non-essential amino acids.

6.4 Product Formation

The objective of cell culture is not to produce cell mass but to synthesize a protein. In the hybridoma cultures, the product is a monoclonal antibody. The concentration profiles of the product in batch and fed-batch cultures are shown
Figure 6.19 Concentrations of non-essential amino acids in a bioreactor fed-batch culture (FB3) of hybridoma cells.
in Figure 6.20. Because of the increased viable cell density and culture span, product concentrations in the fed-batch cultures were substantially higher than that in the batch culture. In FB5, a final antibody concentration of 2400 mg/L was achieved. The total antibody production in FB5 over the 550 hour culture course was calculated to be 4.4 gram. As compared to the batch, a 10-fold increase in volumetric productivity was achieved in FB5.

The specific antibody production rates (Figure 6.21) are shown to be constant for the entire cultivation process through the analysis of the integrated viable cells with time. This computational method is quite reliable since the experimental error is not amplified in the calculation. On the other hand, both constant and variable specific production rates have been reported by many other investigators (Change et al., 1995b; Hiller et al., 1993; Lindardos et al., 1991; Martens et al., 1993; Robinson and Memmert, 1991; Yang et al., 1996). In some cases, the change in specific production rate could be caused by a short culture span, experimental errors, and the method employed for calculation. It should be realized that the specific production rate is a unique characteristic of a specific cell line, and hence could be different from cell line to cell line. The specific antibody production rate in the batch and two fed-batch cultures is essentially the same, indicating that it is not growth associated since the growth rate in these experiments were different. It should be emphasized that the cell line used in this experiment is not specifically selected or genetically altered to increase the specific antibody production rate.
Figure 6.20 Comparison of the product concentration in batch and fed-batch cultures of hybridoma cells.
Figure 6.21 Specific product synthesis rate in batch and fed-batch cultures of hybridoma cells.
6.5 Summary

The results of the hybridoma cultures are summarized in Table 6.1. As a comparison, the two fed-batch cultures conducted with the same hybridoma cell line using concentrated complete media for feeding are also summarized in Table 6.1. Raw data were extracted from figures presented by Glacken (1987) and Chang (1994), and recalculated using the method described in Chapter 3 to be comparable with the results of FB3 and FB5.

Significant increases in viable cell density were achieved through the feeding of concentrated supplemental media in all fed-batch cultures except the one conducted by Glacken. This verifies that the amount of nutrients in basal media is insufficient. The highest viable cell density was attained in FB5 where stoichiometric medium design and an optimal feeding strategy were employed along with the addition of serum and trace metals. This supports the assumption that depletion of one essential nutrient could limit cell growth. The addition of balanced supplemental media resulted in a substantial increases in culture span as shown by FB3 and FB5.

The control of the glucose, glutamine and essential amino acids in FB3 and FB5 led to very low specific lactate production rates. The specific lactate production rate in the fed-batch conducted by Glacken (1987) was lower than that of the batch, but substantially higher than that of FB3 and FB5. This improvement in
Table 6.1: Comparison of culture performance among batch and fed-batch cultures of hybridoma CRL-1606 cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>CRL-1606 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum viable cell density</td>
<td>10^6 cells/ml</td>
<td>2.3</td>
</tr>
<tr>
<td>Maximum Viable Cell Density</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Lactate Concentration</td>
<td>mmol/L</td>
<td>3.2</td>
</tr>
<tr>
<td>Final Ammonia Concentration</td>
<td>mmol/L</td>
<td>1.1</td>
</tr>
<tr>
<td>Ratio of lactate to ammonia</td>
<td>mole/mole</td>
<td>1.3</td>
</tr>
<tr>
<td>Specific Iactate Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.3</td>
</tr>
<tr>
<td>Specific NH3 Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.2</td>
</tr>
<tr>
<td>Ratio of ammonia to cell</td>
<td>mole/mole</td>
<td>0.3</td>
</tr>
<tr>
<td>Ratio of ammonia to glutamine</td>
<td>mole/mole</td>
<td>0.2</td>
</tr>
<tr>
<td>Specific Antibody Production Rate</td>
<td>10^9 mg/...</td>
<td>1.2</td>
</tr>
<tr>
<td>Antibody Concentration</td>
<td>mg/L</td>
<td>0.3</td>
</tr>
<tr>
<td>Specific Antibody Production Rate</td>
<td>10^9 mg/...</td>
<td>0.1</td>
</tr>
<tr>
<td>Ratio of ammonia to glutamine</td>
<td>mole/mole</td>
<td>0.0</td>
</tr>
<tr>
<td>Specific Iactate Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.0046</td>
</tr>
<tr>
<td>Specific NH3 Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.022</td>
</tr>
<tr>
<td>Ratio of ammonia to cell</td>
<td>mole/mole</td>
<td>0.19</td>
</tr>
<tr>
<td>Specific Iactate Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.0016</td>
</tr>
<tr>
<td>Specific NH3 Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.016</td>
</tr>
<tr>
<td>Ratio of ammonia to cell</td>
<td>mole/mole</td>
<td>0.0</td>
</tr>
<tr>
<td>Specific Iactate Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.0046</td>
</tr>
<tr>
<td>Specific NH3 Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.0028</td>
</tr>
<tr>
<td>Ratio of ammonia to cell</td>
<td>mole/mole</td>
<td>0.0</td>
</tr>
<tr>
<td>Specific Iactate Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.0001</td>
</tr>
<tr>
<td>Specific NH3 Production Rate</td>
<td>10^9 mole/cell-h</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ratio of ammonia to cell</td>
<td>mole/mole</td>
<td>0.0</td>
</tr>
</tbody>
</table>

FB3 and FB5 was attributed to the balanced supplemental medium and the feed-on-demand strategy for nutritional control. The specific lactate production rate in the fed-batch conducted by Chang (1994) is not available due to insufficient raw data, but it is perceivable to be higher than that of the batch because of the high lactate production (217 mM), probably caused by absence of any nutritional control. The low lactate to glucose ratio in FB5 indicates that majority of the glucose consumption was utilized for essential roles such as cell mass synthesis and energy production. In both FB3 and FB5, less than 10% of the glucose was converted into lactate, in comparison to 82% in the normal batch (B4) and 46% in a glucose and glutamine limited hybridoma fed-batch culture (Ljunggren and Haggstrom, 1994). It is therefore conclusive that the accumulation of lactate can be solved by the methodology described in this thesis.

The ammonia production was also greatly reduced through the stoichiometric medium design and nutritional control. This is shown by a 5.7-fold decrease in the specific ammonia production rate in FB5 and a 3-fold decrease in the molar ratio of ammonia to glutamine as compared to those of the batch (B4). The specific ammonia production rate reported by Glacken (1987) was considerably higher than that of the batch (B4). Even though the specific production rate was significantly reduced, the final ammonia concentration still reached 13.9 mM in FB5 of which about half was introduced from the supplemental medium due to the non-enzymatic glutamine degradation during storage. Accumulation of ammonia was still problematic. Other approaches to reduce ammonia formation should be taken in order to further improve the culture performance.
The final antibody titer reached 2400 mg/L in FB5, an increase of 45 fold and 2.6 fold compared to that of B4 and FB3, respectively. The productivity was also significantly increased. It should be emphasized that the cell line used in these experiments was not specifically selected or genetically altered. The specific antibody production rate was shown to be constant in each culture and is about the same among B4, FB3, and FB5. The slight difference was probably caused by measurement errors. The average specific antibody production rate is about 0.8 mg/cell-h. A significant high value was calculated from raw data presented by Glacken (1987) for the same cell line (Figure 6.22), presumably caused by the different assay protocol employed for the antibody measurement. If this factor is considered, the final product concentration in this culture should be corrected to be about 120 mg/L. On the other hand, a lower value of production rate was reported by Chang (1994) also for the same hybridoma cell line. No sound explanation is available. Nevertheless, it can be concluded that the enormous increase in the antibody titer was due to the increased time integral of viable cells through the integrated approach for medium design and nutritional control. In combination of genetic approaches to enhance the specific antibody production rate, the product titer can be further improved.

In conclusion, the hybridoma fed-batch cultures using the integrated approach for nutritional control led to significant improvement in culture performance: stable nutritional environment, low by-product formation, high cell densities,
Fed-batch (Glacken, 1987)

$q_p = 1.2 \text{ pg/cell-h}$

Figure 6.22 Specific product synthesis rate in a bioreactor fed-batch culture of hybridoma cells using a predetermined feeding strategy (Glacken, 1987).
high product concentration, and high productivity. This verifies the application of the approach devised in the thesis for the optimization of animal cell culture process.
7. Material and Energy Balances in Hybridoma Cultures

7.1 Material Balance Model

7.1.1 Metabolic Reaction Network

A metabolic reaction network has been described in detail for the determination of the stoichiometric equation (see Chapter 4). In the medium design, the catabolism of essential amino acids (except glutamine) was assumed to be negligible as compared to their consumption for protein synthesis. In the following material balance studies, the catabolism of five essential amino acids (isoleucine, leucine, lysine, threonine, and valine) is considered (Figure 7.1) to verify the above assumption. Through a previously described lumping approach, the catabolic pathways of these amino acids were also simplified as single reactions. The reactions involved in the metabolic network are listed in Table 7.1. Only the relevant reactants and products are shown for the sake of simplicity. The reactions for protein synthesis and nucleotide synthesis have been described in Chapter 4.

7.1.2 Metabolic Reaction Extents

The reaction extents can be determined from extracellular measurements and cell composition. In the following analysis, it is assumed that there is no net
Figure 7.1 Schematic diagram of the metabolic reaction network of animal cells
Table 7.1 Major reactions in the animal cell metabolic reaction network.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Overall Stoichiometry of Reaction</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_1$</td>
<td>Glucose + 2ADP + 2NAD$^+$ + 2P$\rightarrow$ 2Pyruvate + 2ATP + 2NADH + 2H$^+$ + 2H$_2$O</td>
<td>(7.1)</td>
</tr>
<tr>
<td>$F_2$</td>
<td>Pyruvate + NADH + H$^+$ $\rightarrow$ Lactate + NAD$^+$</td>
<td>(7.2)</td>
</tr>
<tr>
<td>$F_3$</td>
<td>Acetyl-CoA + Oxaloacetate + H$_2$O $\rightarrow$ Citrate + CoA + H$^+$</td>
<td>(7.3)</td>
</tr>
<tr>
<td>$F_4$</td>
<td>Pyruvate + Glutamate $\rightarrow$ Alanine + $\alpha$-Ketoglutarate</td>
<td>(7.5)</td>
</tr>
<tr>
<td>$F_{5,a}$</td>
<td>Glutamate + NAD$^+$ $\rightarrow$ $\alpha$-Ketoglutarate + NADH + NH$_3$</td>
<td>(7.6)</td>
</tr>
<tr>
<td>$F_6$</td>
<td>Glutamine + H$_2$O $\rightarrow$ Glutamate + NH$_3$</td>
<td>(7.7)</td>
</tr>
<tr>
<td>$F_7$</td>
<td>Glutamate + ATP + 2NADPH $\rightarrow$ Proline + ADP + NADP$^+$ + H$_2$O</td>
<td>(7.8)</td>
</tr>
<tr>
<td>$F_8$</td>
<td>Oxaloacetate + Glutamate $\rightarrow$ Aspartate + $\alpha$-Ketoglutarate</td>
<td>(7.9)</td>
</tr>
<tr>
<td>$F_9$</td>
<td>Aspartate + Glutamine $\rightarrow$ Asparagine + Glutamate</td>
<td>(7.10)</td>
</tr>
<tr>
<td>$F_{10}$</td>
<td>Oxaloacetate $\rightarrow$ Pyruvate + CO$_2$</td>
<td>(7.11)</td>
</tr>
<tr>
<td>$F_{11}$</td>
<td>Glucose + 2 Glutamate + 4 NAD$^+$ $\rightarrow$ 2 Serine + 2 $\alpha$-Ketoglutarate + 4NADH + 4H$^+$</td>
<td>(7.12)</td>
</tr>
<tr>
<td>$F_{12}$</td>
<td>Serine + Tetrahydrofolate $\rightarrow$ Glycine + N$^+$,N$^{\alpha}$-Methylenetetrahydrofolate</td>
<td>(7.13)</td>
</tr>
<tr>
<td>$F_{13}$</td>
<td>Pyruvate + NAD$^+$ + CoA $\rightarrow$ Acetyl-CoA + NADH + CO$_2$</td>
<td>(7.14)</td>
</tr>
<tr>
<td>$F_{14}$</td>
<td>Lysine + 5NAD$^+$ + FAD + CoA $\rightarrow$ 2Acetyl-CoA + 5NADH + FADH$_2$ + 2CO$_2$ + 2NH$_3$</td>
<td>(7.15)</td>
</tr>
<tr>
<td>$F_{15}$</td>
<td>Isoleucine + 3NAD$^+$ + FAD + ATP + 2CoA $\rightarrow$ Succinyl-CoA + Acetyl-CoA + 3NADH + FADH$_2$ + ADP + NH$_3$</td>
<td>(7.16)</td>
</tr>
<tr>
<td>$F_{16}$</td>
<td>Leucine + 2NAD$^+$ + FAD + 2ATP $\rightarrow$ 3 Acetyl-CoA + 2NADH + FADH$_2$ + 2ADP + NH$_3$</td>
<td>(7.17)</td>
</tr>
<tr>
<td>$F_{17}$</td>
<td>Glucose + ATP + 2NADP$^+$ + H$_2$O $\rightarrow$ Ribose-5-P + 2NADPH + ADP + CO$_2$ + 2H$^+$</td>
<td>(7.18)</td>
</tr>
<tr>
<td>$F_{18}$</td>
<td>Glucose + Glycogen$<em>n$ + 2 ATP $\rightarrow$ Glycogen$</em>{n+2}$ + 2 ADP + 2 P,</td>
<td>(7.19)</td>
</tr>
<tr>
<td>$F_{19}$</td>
<td>Glucose + 4 ATP + 2 NADPH + 2 NAD$^+$ (Fatty Acids)$<em>{2n}$ $\rightarrow$ (Fatty Acids)$</em>{2n+2}$ + 2 NADH + 4ADP + 2NADP$^+$ + 2CO$_2$</td>
<td>(7.20)</td>
</tr>
<tr>
<td>$F_{20}$</td>
<td>Glucose + ATP + 12NADP$^+$ + 7H$_2$O $\rightarrow$ 12NADPH + ADP + 6CO$_2$ + 12H$^+$ + P$_i$</td>
<td>(7.21)</td>
</tr>
<tr>
<td>$F_{21}$</td>
<td>Threonine + ATP + NAD$^+$ + CoA $\rightarrow$ Succinyl-CoA + NADH + ADP + NH$_3$</td>
<td>(7.22)</td>
</tr>
<tr>
<td>$F_{22}$</td>
<td>Valine + ATP + 4NAD$^+$ + FAD + CoA $\rightarrow$ Succinyl-CoA + 4 NADH + FADH$_2$ + ADP + CO$_2$ + NH$_3$</td>
<td>(7.23)</td>
</tr>
<tr>
<td>$F_{23}$</td>
<td>$\alpha$-Ketoglutarate + NAD$^+$ + CoA $\rightarrow$ Succinyl-CoA + NADH + CO$_2$</td>
<td>(7.24)</td>
</tr>
<tr>
<td>$F_{24}$</td>
<td>Succinyl-CoA + GDP + FAD + P$_i$ $\rightarrow$ Fumarate + GTP + FADH$_2$ + CoA</td>
<td>(7.25)</td>
</tr>
<tr>
<td>$F_{25}$</td>
<td>Fumarate + NAD$^+$ + H$_2$O $\rightarrow$ Oxaloacetate + NADH + H$^+$</td>
<td>(7.26)</td>
</tr>
</tbody>
</table>
accumulation of nutrients, product, by-products, or intermediates in cells.

The consumption of each amino acid on a per cell basis is defined by eq. (7.27).
The stoichiometric demand for the syntheses of cellular proteins, product, and
nucleotides has been defined in Chapter 4 [eq. (4.43)].

\[ U_{a,i} = \frac{A}{\Delta N_i} \quad \text{(for each of the 20 amino acids)} \quad (7.27) \]

The reaction extents for the syntheses of carbohydrates \( F_{18} \), lipids \( F_{19} \),
riboses \( F_{17} \), and reducing power \( F_{20} \) from glucose have also been
determined in Chapter 4 by equations (4.44), (4.45), (4.46), and (4.49),
respectively. Material balances of glycine and serine are employed to solve \( F_{11} \)
and \( F_{12} \) according to eqs. (7.28) and (7.29), respectively. Since \( F_{11} \) is defined as
the amount of glucose used for serine synthesis, the amount of serine
synthesized is, therefore, twice \( F_{11} \). Similarly, a material balance on glucose is
used to calculate \( F_1 \) according to equation (7.30).

\[ F_{11} = \left[ F_{12} + \theta_{\text{ser}}^p + \theta_{\text{ser}}^{\text{nuc}} - U_{\text{ser}} \right] / 2 \quad (7.28) \]
\[ F_{12} = \theta_{\text{gly}}^p + \theta_{\text{gly}}^{\text{nuc}} - U_{\text{gly}} \quad (7.29) \]
\[ F_1 = \frac{\Delta G_{\text{Glc}}}{\Delta N_i} - \left[ F_{11} + F_{17} + F_{18} + F_{19} + F_{20} \right] \quad (7.30) \]

Equations (7.31) to (7.41) are derived from the material balances of amino
acids. In the case of non-essential amino acids (NAA), three factors need to be
considered. These are the requirements for protein (including product) and
nucleotide syntheses, the consumption (or production if negative) from the
medium, and the biosynthesis from glutamine. For essential amino acids, the
requirement for protein synthesis, the consumption from medium, and the
catabolism (only for five major amino acids) are considered in the material
balances. The reaction extent, $F_5$, is calculated using a carbon balance on
 glutamate. A nitrogen balance on glutamate is used to determine $F_{5,a}$ by
subtracting $F_4$, $F_8$, and $F_{11}$ from $F_5$.

$$F_4 = \theta_{ux}^p - U_{ux}$$

(7.31)

$$F_8 = U_{glu} - \theta_{glu}^p - \theta_{glu}^{nuc}$$

(7.32)

$$F_7 = \theta_{pro}^p - U_{pro}$$

(7.33)

$$F_2 = F_8 + U_{glu} - \theta_{glu}^p - \theta_{glu}^{nuc} - F_7$$

(7.34)

$$F_6 = \theta_{asn}^p - U_{asn}$$

(7.35)

$$F_8 = F_5 + \theta_{asp}^p + \theta_{asp}^{nuc} - U_{asp}$$

(7.36)

$$F_{14} = U_{lys} - \theta_{lys}^p$$

(7.37)

$$F_{15} = U_{lle} - \theta_{lle}^p$$

(7.38)

$$F_{16} = U_{leu} - \theta_{leu}^p$$

(7.39)

$$F_{21} = U_{thr} - \theta_{thr}^p$$

(7.40)

$$F_{22} = U_{val} - \theta_{val}^p$$

(7.41)

By assuming that intracellular intermediates do not accumulate in cells, a
material balance on each intermediate then gives the following equations:

$$F_{23} = F_5 + F_3$$

(7.42)

$$F_{24} = F_{15} + F_{21} + F_{22} + F_{23}$$

(7.43)

$$F_{26} = F_{24} + F_{23}$$

(7.44)
\begin{align*}
F_{10} &= F_{25} - F_3 - F_1 \\
F_{13} &= 2F_1 + F_{10} - F_2 - F_4 \\
F_3 &= F_{13} + 2F_{14} + F_{15} + 3F_{16}
\end{align*}

(7.45) \quad (7.46) \quad (7.47)

where \( F_{25} \) is the total amount of fumarate produced from the synthesis of nucleotides as defined by eq. (7.48), and \( F_2 \) is the stoichiometric lactate production as defined by equation (7.49).

\[
F_{25} = 2N_A + N_0
\]

(7.48)

\[
F_2 = \frac{\Delta L_{ac}}{\Delta N_i}
\]

(7.49)

By adding eqs. (7.42) to (7.47), \( F_3 \) can be solved:

\[
F_3 = 2F_1 - F_2 - F_4 + F_5 - F_6 + 2F_{14} + 2F_{15} + 3F_{16} + F_{21} + F_{22} + F_{25}
\]

(7.50)

The above analysis correlates the cell composition, nutrient consumption, product formation, and by-product formation with the reaction extents. With experimentally measured data, the metabolic reaction network is now completely defined by equations (4.27) to (4.50).

### 7.1.3 Fractional Contribution Model

Figure 7.2 is the schematic of a fractional contribution model: a substrate has multiple sources, and is converted into multiple products through independent pathways. The contribution of source \( i \) to product \( j \) is defined by equation (7.51).

This model is repeatedly used in the following material balances.

\[
\delta_{i,j} = \frac{\eta_i - \phi_i}{\sum_{i=1}^n \eta_i}
\]

(7.51)

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Figure 7.2 Schematic diagram of a fractional contribution model
7.2 Material Balance Results and Discussions

Data obtained from the three hybridoma fed-batch (FB1, FB2, and FB3) and one batch (B4) cultures are employed in the following material balances. Data calculated from measurements from the above four experiments (see Chapter 6) are listed in Table 7.2. These data, together with the cell composition, are employed in the above outlined material balance model for the determination of the reaction extents (Table 7.3). Viable and dead cells may have different cellular composition. However, it is assumed that the difference in cellular composition is caused by post-death events such as the loss of cell membrane integrity or lysis of dead cells.

7.2.1 Balances of Essential Amino Acids

Material balances on essential amino acids are based on the calculated stoichiometric demands for protein synthesis ($\theta_{s,i}$) and the measured consumption. The amount of each essential amino acid required for the syntheses of cellular proteins and product can be calculated from the stoichiometric model. The actual stoichiometric consumption on a per cell basis can be calculated from the measurements of the total consumption and net increase in total cell number (Table 7.2). The differences between the two parameters, shown as percentages of $\theta_{s,i}$, are presented in Table 7.4. The stoichiometric demand of each amino acid for protein synthesis varies only slightly among the four experiments due to the differences in the specific product synthesis rate. On the other hand, the specific amino acid consumption
Table 7.2. Extracellular measurements from batch and fed-batch cultures of hybridoma cells

<table>
<thead>
<tr>
<th></th>
<th>FB1</th>
<th>FB2</th>
<th>FB3</th>
<th>B4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product formation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta N_i$ (cells)</td>
<td>4.46x10^4</td>
<td>2.00x10^4</td>
<td>2.35x10^4</td>
<td>3.19x10^4</td>
</tr>
<tr>
<td>Antibody (mg)</td>
<td>9.03</td>
<td>8.09</td>
<td>925</td>
<td>56</td>
</tr>
<tr>
<td>$\mu$ (1/h)</td>
<td>0.016</td>
<td>0.012</td>
<td>0.023</td>
<td>0.033</td>
</tr>
<tr>
<td>By-product formation</td>
<td>$\mu$mole</td>
<td>$\mu$mole</td>
<td>mmole</td>
<td>mmole</td>
</tr>
<tr>
<td>Lac</td>
<td>1594.0</td>
<td>673.0</td>
<td>9.48</td>
<td>35.89</td>
</tr>
<tr>
<td>NH$_3$</td>
<td>206.0</td>
<td>85.0</td>
<td>5.07</td>
<td>2.53</td>
</tr>
<tr>
<td>Nutrient consumption*</td>
<td>$\mu$mole</td>
<td>$\mu$mole</td>
<td>mmole</td>
<td>mmole</td>
</tr>
<tr>
<td>GIC</td>
<td>1635.0</td>
<td>736.0</td>
<td>51.79</td>
<td>22.08</td>
</tr>
<tr>
<td>Glu</td>
<td>384.0</td>
<td>204.4</td>
<td>17.01</td>
<td>4.52</td>
</tr>
<tr>
<td>Ile</td>
<td>33.9</td>
<td>18.8</td>
<td>2.42</td>
<td>0.51</td>
</tr>
<tr>
<td>Leu</td>
<td>60.2</td>
<td>32.2</td>
<td>4.39</td>
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</tr>
<tr>
<td>Lys</td>
<td>66.5</td>
<td>30.4</td>
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<td>Met</td>
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<td>7.8</td>
<td>1.08</td>
<td>0.12</td>
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<td>Phe</td>
<td>22.3</td>
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</tr>
<tr>
<td>Thr+Arg</td>
<td>100.4</td>
<td>40.8</td>
<td>4.75</td>
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<td>Tyr</td>
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<td>11.0</td>
<td>1.18</td>
<td>0.15</td>
</tr>
<tr>
<td>Val+Trp</td>
<td>49.5</td>
<td>29.6</td>
<td>3.50</td>
<td>0.44</td>
</tr>
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<td>Ala</td>
<td>-134.7</td>
<td>-40.0</td>
<td>-3.88</td>
<td>-1.88</td>
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<tr>
<td>Asn</td>
<td>26.8</td>
<td>-1.0</td>
<td>2.00</td>
<td>0.07</td>
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<td>82.1</td>
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<td>-1.95</td>
<td>-0.55</td>
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<tr>
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<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Ser</td>
<td>34.8</td>
<td>12.4</td>
<td>0.80</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Negative values signify net production
Table 7.3 Reaction extents of the hybridoma metabolic reaction network determined from extracellular measurements

<table>
<thead>
<tr>
<th>Reaction</th>
<th>FB1*</th>
<th>FB2*</th>
<th>FB3*</th>
<th>B4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>2.92</td>
<td>2.87</td>
<td>1.43</td>
<td>6.10</td>
</tr>
<tr>
<td>F2</td>
<td>3.57</td>
<td>3.37</td>
<td>0.40</td>
<td>11.25</td>
</tr>
<tr>
<td>F3</td>
<td>2.51</td>
<td>2.69</td>
<td>2.67</td>
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</tr>
<tr>
<td>F4</td>
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<td>0.36</td>
<td>0.32</td>
<td>0.73</td>
</tr>
<tr>
<td>F5</td>
<td>0.53</td>
<td>0.71</td>
<td>0.33</td>
<td>0.96</td>
</tr>
<tr>
<td>F5,a</td>
<td>-0.04</td>
<td>-0.02</td>
<td>-0.14</td>
<td>-0.16</td>
</tr>
<tr>
<td>F6</td>
<td>0.75</td>
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<td>0.61</td>
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<tr>
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<td>0.09</td>
</tr>
<tr>
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<td>0.01</td>
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<td>0.02</td>
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</tr>
<tr>
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<td>0.00</td>
<td>0.03</td>
<td>0.04</td>
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</tr>
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<td>2.44</td>
</tr>
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<td>F25</td>
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<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>F26</td>
<td>3.09</td>
<td>3.44</td>
<td>3.05</td>
<td>2.46</td>
</tr>
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</table>

* all units are mmole/10⁹ cells
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<tr>
<th>Amino Acids</th>
<th>Val/Tyr</th>
<th>Thr/Arg</th>
<th>Phe/Met</th>
<th>Leu/Lys</th>
<th>Ile/Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>10.9</td>
<td>10.9</td>
<td>10.9</td>
<td>10.9</td>
<td>10.9</td>
</tr>
</tbody>
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Table 7.4: Material balances on essential amino acids in hybridoma batch and fed-batch cultures.
rates differ significantly among the four experiments (data not shown) due to
the differences in the growth rate. This suggests that the stoichiometric
parameters are generic but the metabolic fluxes are not. Except for isoleucine
and leucine, the differences between the measured consumption and the
calculated demand for protein synthesis are insignificant (in the range of error).
A significant amount of isoleucine and leucine was metabolized, probably for
energy production or lipid synthesis, in both FB3 and B4, but not in FB1 or FB2.
The same initial medium with very low amino acid concentrations was used in
both FB1 and FB2 (see Chapter 3). On the other hand, IMDM was used in B4
and a different initial medium was used in FB3. Both media have higher amino
acid concentrations. These results suggest that isoleucine and leucine (maybe
some other essential amino acids) could be used for energy production or as
precursors for lipid synthesis if an excess amount is available to the cells. These
results are consistent with those found by other investigators (Chang et al.,
1995; Hiller et al., 1994; Ljunggren and Haggstrom, 1992). The above results
also support the assumption employed in the supplemental medium design that
the essential role of these amino acids is for protein synthesis. Energy could be
derived from these essential amino acids, but this is undesirable because of the
associated ammonia production.

When the measured stoichiometric consumption is plotted versus the calculated
stoichiometric demand for protein synthesis (Figure 7.3), most of the data points
are close to the diagonal line. If a data point falls in the bottom-right region, it is
most likely caused by an error since animal cells cannot synthesize these
essential amino acids. On the other hand, a data point in the top-left region
Figure 7.3 Relationship between the measured stoichiometric amino acid consumption and the calculated amino acid demand for cellular protein and product synthesis.
indicates possible utilization for purposes other than protein synthesis (or an error if close to the line). One such data point (isoleucine in B4) is marked with a rectangle in the figure. Comparison among the essential amino acids shows that methionine, phenylalanine, and tyrosine are consistently close to the line but others are more scattered. This suggests that the consumption of these three amino acids could be employed to accurately estimate the production of cell mass under various culture conditions.

7.2.2 Balances of Non-essential Amino Acids

Material balances on NAAs include three factors: the stoichiometric demands for protein and nucleotide syntheses, the uptake from culture medium or net production, and the synthesis from glutamine and other essential amino acids. Animal cells can produce all seven non-essential amino acids. The direct nitrogen donor is glutamate derived from glutamine and other essential amino acids. Based on material balances, the amount of NAAs synthesized (Table 7.5) can be calculated using equation (7.52).

\[ S_{a,i} = \theta_{a,i}^p + \theta_{a,i}^{nuc} - U_{a,i} \]  (non-essential amino acids)  (7.52)

A negative value in the NAA uptake shows a net amount of secretion into the culture medium. Alanine was always produced in all four cultures. In FB3, both glucose and glutamine were well controlled, and alanine production was the lowest among the four experiments. In FB2, glutamine was limited but glucose was in excess. Alanine production was also very low. These results suggest that alanine production is related with the availability of both glucose and
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<td>Asn</td>
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<td></td>
<td></td>
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</tr>
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</tr>
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<tr>
<td>Total</td>
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<td>0.72</td>
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</tr>
<tr>
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<td></td>
</tr>
</tbody>
</table>

Table 7.5: Material balances on nonessential amino acids in hybridoma batch and Fed-batch cultures.
glutamine. This is supported by the fact that much higher alanine production was observed in both FB1 and FB4 where both glucose and glutamine were in excess. Similar to alanine, glutamate was usually produced since a small net secretion into the culture medium was observed in all four cultures. Other non-essential amino acids showed moderate synthesis and uptake, depending on the culture conditions. The reaction extent of glycine production from serine ($F_{12}$) is very small (0.004 mmole/10⁹ cells) in FB1 as shown in Table 7.5 (this parameter is shown as zero in Table 7.3 due to the two significant digit numbers used). The reaction extent of $F_{12}$ may be underestimated due to the nature of the calculation [eq. (7.28)], because the flux of serine to glycine may be insignificant as compared to the flux of glycine for protein and nucleotide syntheses. It should be noted that this reaction also provides the essential one carbon units for biosyntheses. However, the reaction extent of $F_{12}$ does not reflect the actual flux of one carbon unit metabolism because there are alternative pathways, such as the methyl cycle where choline provides the one carbon units.

7.2.3 Nitrogen Balance on Glutamine

One of the essential roles of glutamine in animal cell metabolism is to provide nitrogen for nucleotide synthesis in addition to protein synthesis. There are two nitrogen atoms in glutamine, but only the one in the amide group is used for nucleotide synthesis. The amino group and the carbon skeleton remains as glutamate. Glutamine can also be hydrolyzed into ammonia and glutamate via glutaminase. There are different fates for the glutamine-derived-glutamate. It
can be directly incorporated into proteins or converted into proline. Its carbon skeleton can enter the TCA cycle, while the amino group is transaminated to form other NAAs via transaminases or converted into ammonia via glutamate dehydrogenase. Glutamate dehydrogenase activity was detected in Hybridoma CRL-1606, the same cell line used in this work, in NMR spectroscopy studies by Zupke and coworkers (1995b). Both negative and positive fluxes from glutamate to α-ketoglutarate were found under different culture conditions.

The nitrogen requirement for NAA synthesis can be calculated from the total NAA production. The glutamine-derived-glutamate, joined by that generated from other essential amino acids, is used as the direct nitrogen source for the synthesis of other NAAs via transaminases. If the total amount of glutamate produced is less than the total demands for NAA synthesis (this indicates free ammonia is consumed for NAA synthesis), no ammonia will be produced from glutamate. Otherwise, the extra amount of the amino group is converted into ammonia via glutamate dehydrogenase. The contribution of glutamine or other essential amino acids for NAA synthesis or ammonia production is then calculated using the fractional contribution model. The production of ammonia from glutamine is calculated by subtracting the contribution of other essential amino acids from the measured total ammonia production per cell. The difference between the total nitrogen consumption from glutamine and the total nitrogen output is reported as an error.

The results from the glutamine nitrogen balance are listed in Table 7.6. The total consumption of glutamine nitrogen differs significantly from batch to fed-batch,
Table 7.6. Material balances on glutamine and glucose in hybridoma cultures

<table>
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<th>FB1</th>
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<th>FB3</th>
<th>B4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^9 mmol/cell</td>
<td>%</td>
<td>10^9 mmol/cell</td>
<td>%</td>
</tr>
<tr>
<td><strong>Glutamine</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Total Nitrogen</strong></td>
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<td>1.448</td>
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<tr>
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<td>3.7</td>
<td>0.065</td>
<td>3.2</td>
</tr>
<tr>
<td>NAA</td>
<td>0.737</td>
<td>42.3</td>
<td>0.959</td>
<td>46.9</td>
</tr>
<tr>
<td>NH3</td>
<td>0.462</td>
<td>26.8</td>
<td>0.425</td>
<td>20.8</td>
</tr>
<tr>
<td>Error</td>
<td>0.239</td>
<td>13.9</td>
<td>0.355</td>
<td>17.4</td>
</tr>
<tr>
<td><strong>Carbon Skeleton</strong></td>
<td>0.861</td>
<td>1.022</td>
<td>0.724</td>
<td>1.416</td>
</tr>
<tr>
<td>Proteins*</td>
<td>0.110</td>
<td>12.7</td>
<td>0.120</td>
<td>11.7</td>
</tr>
<tr>
<td>TCA Cycle</td>
<td>0.531</td>
<td>61.7</td>
<td>0.705</td>
<td>69.0</td>
</tr>
<tr>
<td>Glu &amp; Pro</td>
<td>0.221</td>
<td>25.6</td>
<td>0.197</td>
<td>19.3</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3.666</td>
<td>3.680</td>
<td>2.204</td>
<td>6.923</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>0.049</td>
<td>1.3</td>
<td>0.049</td>
<td>1.3</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.536</td>
<td>14.6</td>
<td>0.536</td>
<td>14.6</td>
</tr>
<tr>
<td>DNA &amp; RNA</td>
<td>0.036</td>
<td>1.0</td>
<td>0.036</td>
<td>1.0</td>
</tr>
<tr>
<td>Serine</td>
<td>0.043</td>
<td>1.2</td>
<td>0.106</td>
<td>2.9</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.081</td>
<td>2.2</td>
<td>0.081</td>
<td>2.2</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.787</td>
<td>48.8</td>
<td>1.682</td>
<td>45.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.223</td>
<td>6.1</td>
<td>0.179</td>
<td>4.9</td>
</tr>
<tr>
<td>TCA Cycle</td>
<td>0.912</td>
<td>24.9</td>
<td>1.011</td>
<td>27.5</td>
</tr>
</tbody>
</table>

* This is the amount of glutamine that is directly incorporated into proteins (the amount of glutamine carbon skeleton or nitrogen incorporated into proteins via non-essential amino acids is not included in this term).
mainly due to the differences in NAA synthesis and ammonia production. The amount of nitrogen used in protein and nucleotide is relatively constant and represents only a small percentage of the total. Interestingly, a relatively constant percentage (about 45%) of the total glutamine nitrogen was used for NAA synthesis in all four experiments, despite the fact that the total glutamine consumption was quite different.

There is an error of about 20% in the glutamine nitrogen balance for all experiments. Detailed analysis shows that glutamate produced from glutamine and other essential amino acids equals approximately the amount of NAA synthesis. This is consistent with the results found by Street and co-workers (1993) that the amino group in glutamine was transaminated for the synthesis of non-essential amino acids. On the other hand, only a part of the ammonia produced from glutamine hydrolysis via glutaminase was found in the culture medium. The rest is reported as the error calculated from the total nitrogen balance. This is probably caused by ammonia evaporation during the culture process, measurement errors, and/or utilization of ammonia/glutamine amide group for synthesis of amino acid analogues, such as ornithine and citrulline (both not measured).

7.2.4 Relationship among Ammonia Production, NAA Synthesis, and Glutamine Utilization

A strong relationship among the total glutamine consumption, the synthesis of NAAs, and the production of ammonia, can be observed in Figure 7.4. It is
Figure 7.4 Relationship among ammonia production, glutamine consumption, and the synthesis of nonessential amino acids
apparent that both ammonia production and NAA synthesis increase with glutamine consumption. Except for alanine and glutamate, other non-essential amino acids in the culture medium can be utilized by cells. Hence sufficient amount of NAAs in the culture medium will reduce the burden of the cells to synthesize NAAs from glutamine. This is supported by the fact that NAA synthesis in FB3 (where sufficient NAAs were fed) is much less than in B4 (where insufficient NAAs were present in IMDM). It is therefore necessary to provide NAAs to cells in order to reduce the glutamine demand which leads to a low ammonia production, even though NAAs are not essential in the sense of supporting cell growth. However, this strategy does not work unless glutamine is controlled at an optimal low concentration. When glutamine is in excess, feeding NAAs to cells may cause them to accumulate and may eventually inhibit their synthesis. This will result in ammonia production via glutamate dehydrogenase since excess amount of glutamine nitrogen cannot be transaminated into NAAs (data not shown). Hence, feeding of NAAs needs to be balanced with the maximum uptake and coupled with glutamine control. These results confirm the hypothesis (in Chapter 4 for the determination of the real reaction network) that maximum NAA uptake leads to minimum ammonia formation under a glutamine-limited condition.

7.2.5 Material Balance on Glutamine Carbon Skeleton

Unlike the glutamine nitrogen metabolism, the metabolism of the glutamine carbon skeleton is simpler. About 8 to 16% of the glutamine is directly incorporated into proteins (Table 7.6). The rest of the glutamine is converted
into glutamate when the amide group is hydrolyzed or utilized for nucleotide synthesis. Some of the glutamine-derived-glutamate is directly incorporated into proteins, and some may be secreted into the culture medium. Another small portion is consumed for proline synthesis. The total percentage of the glutamine carbon skeleton used for the above three purposes varies from 20 to 39% (Table 7.6). The rest of the glutamine carbon skeleton (45-69%) enters the TCA cycle for energy production or the synthesis of other cellular components (such as alanine, lactate, lipids).

7.2.6 Material Balance on Glucose

The total glucose consumption differs significantly among the four experiments, with the greatest difference of 3-fold between FB3 and B4 (Table 7.6). This indicates that cells use glucose (and glutamine, see Table 7.6) much more efficiently under controlled nutritional environment. The consumption of glucose for lipid synthesis is much greater than that for carbohydrates, ribose, and NADPH. The fact that only a very small percentage of glucose (3-8%) was consumed for ribose and NADPH production indicates that the pentose cycle is not very active in animal cell metabolism in vitro. This is consistent with the results found by Lazo (1981) and Sharfstein and co-workers (1994). The contribution of glutamine to alanine production is estimated to be negligible as compared to that of glucose. The estimated percentage of glucose used for serine and alanine synthesis ranges from 7 to 10%. Not surprisingly, about 60% to 82% of glucose was consumed for lactate and energy production. It is very interesting that very little glucose was consumed in the TCA cycle in the batch.
culture (B4) where glucose was in excess, but about 50% of glucose was consumed in the TCA cycle in FB3 where glucose was limited. These data suggest that production of sufficient energy for cell growth even under limited nutrient conditions is the highest priority for living cells.

Previously, it was assumed that production of lactate was due to the over-flow of glucose to pyruvate in the glycolytic pathways. Based on this assumption, a very small lactate to glucose ratio was assumed in the stoichiometric model to design a supplemental medium for feeding (see chapter 4). The glucose concentration was controlled at a very low level as the result of the feeding strategy to reduce lactate production. This assumption was confirmed by the significant difference (81% versus 9%) of glucose consumption leading to lactate production between FB3 and B4.

7.3 Energy Metabolism Model

7.3.1 ATP Production

Figure 7.5 depicts a schematic diagram of the metabolic reaction network that has been described in detail in Chapter 4 and section 7.1. In animal cell culture, both glucose and glutamine have been shown to be the major energy sources (McKeenan, 1986; Sharfstein et al., 1994; Zielke et al., 1984). The majority of glucose is converted into pyruvate through the glycolytic pathway. In some cases, cells rely heavily on this pathway to obtain energy for growth and maintenance. Pyruvate produced from glucose can then be converted into end
Figure 7.5 Contributions of glucose and glutamine to energy metabolism in animal cell metabolic reaction network.
products, such as lactate and alanine, or acetyl-CoA that enters the TCA cycle for energy production. In glutamine metabolism, there are different fates for the two nitrogen atoms and the carbon skeleton. Majority of the glutamine carbon skeleton enters the TCA cycle as α-ketoglutarate. It is also possible that some of the essential amino acids can be used for energy production in addition to protein synthesis. Only five major amino acids are considered in the energy metabolism because the catabolism of others has been shown to be insignificant (see 7.2.1).

Three major energy forms, ATP, NADH, and FADH$_2$, can be generated. A certain amount of ATP and sometimes GTP can be produced directly from the free energy release in some reactions. For instance, the conversion of one glucose molecule into two pyruvate molecules is concomitant with the net production of two ATPs. In some of the reactions, ATP needs to be invested for the production of NADH, such as in the catabolism of essential amino acids. The invested amount needs to be deducted from the total direct ATP production. According to the stoichiometry of reaction network, the net direct ATP production [eq. (7.52)] can be determined. The oxidative phosphorylation of NADH and FADH$_2$ generates most of the ATP required for cell growth. The amount of ATP produced from the phosphorylation of NADH is calculated by multiplying the total NADH with its P/O ratio, as defined by eq. (7.53). The cytosolic NADH generated from the glycolytic pathway is not included in eq. (7.53), because it is generally believed that cytosolic NADH is converted into mitochondrial FADH$_2$ through the glycerol phosphate shuttle. Hence, it is included in the ATP production from the phosphorylation of FADH$_2$ as described by eq. (7.54). It
should be noticed that there are two different pathways for the conversion of glutamate to $\alpha$-ketoglutarate. One is the hydrolysis of glutamate via glutamate dehydrogenase, and the other is the transamination of the amino group via transaminases. Energy is produced in the first pathway, but not in the second one. The total amount of glutamate converted to $\alpha$-ketoglutarate is defined as $F_5$. The amount of glutamate transaminated can be determined from the material balances of the non-essential amino acids. Hence, the amount of glutamate hydrolyzed, denoted as $F_{5,a}$, can be calculated by subtracting the amount of glutamate transaminated from $F_5$. It is possible that $F_{5,a}$ can be negative, which signifies that free ammonia is assimilated into glutamate via the reverse reaction, and hence NADH is consumed. The total ATP production, defined by eq. (7.56), is then the sum of the ATP produced from the above three different energy forms.

$$ATP_p^d = 2F_1 + F_{24} - [F_7 + F_{15} + 2F_{16} + F_{21} + F_{22}]$$  \hspace{1cm} (7.52)

$$ATP_p^N = [F_3 + F_{5,a} + 4F_{11} + 4F_{16} + 3F_{15} + 2F_{16} + 4F_{21} + 4F_{22} + 4F_{24}] \eta_N$$  \hspace{1cm} (7.53)

$$ATP_p^f = [2F_1 - F_4 + F_{13} + 5F_{15} + 3F_{16} + 2F_{21} + 4F_{22} + 4F_{24}] \eta_f$$  \hspace{1cm} (7.54)

$$F_{5,a} = F_5 - F_{4} - F_{8} - 2F_{11}$$  \hspace{1cm} (7.55)

$$ATP_p^N = ATP_p^d + ATP_p^N + ATP_p^f$$  \hspace{1cm} (7.56)

In order to gain more insights into energy metabolism, it is desirable to know the contribution of each nutrient to the ATP production. It has been reported in many cases that the glutamine carbon skeleton can be incorporated into different metabolites such as aspartate, alanine, lactate, and lipids, via the malate to pyruvate shunt (McKeehan, 1986; Mancuso et al., 1994; Sharfstein et al., 1994).
Certainly, a portion of the glutamine carbon is converted into CO$_2$ in the TCA cycle. Similarly, glucose is converted into pyruvate via the glycolytic pathway, and then enters the TCA cycle as acetyl-CoA. Therefore, both glucose and glutamine can contribute to all of the intermediates and end-products as shown in Figure 7.5. The amount of glucose entering the TCA cycle depends on the nutritional environment, as shown previously by the material balances. The fact that a significant part of the glucose and glutamine metabolic pathways overlaps renders the problem very complicated.

The fractional contribution model is employed here to solve the above problem. The principle of this model is the assumption that substrates derived from different sources possess an equal probability to be used for the formation of a particular product. Hence, the contribution of the original source to the product depends on the percentage of the source in the total substrate pool. This principle is universally true if these substrates are identical (cells may view free and enzyme bound substrates differently) and if there is a common pool for each substrate.

The catabolism of the five essential amino acids considered in the material balances is assumed only for energy production. This will not affect the results in any significant way, since the amount of the TCA cycle intermediates derived from these amino acids is trivial as compared to those derived from glucose and glutamine. Hence, only the fractions of glucose and glutamine are shown in the model schematic (Figure 7.5). These fractions can be determined on the basis of material balances and the fractional contribution principle, according to eqs.
(7.57) to (7.73). Two cases are considered for \( F_8 \) since aspartate can either be produced from or be converted into oxaloacetate as shown by the material balances.

\[
X_3 = \frac{X_7 F_3 + F_5}{F_{23}} \quad (7.57)
\]

\[
Y_3 = \frac{Y_7 F_3}{F_{23}} \quad (7.58)
\]

\[
X_4 = \frac{X_7 F_{23}}{F_{24}} \quad (7.59)
\]

\[
Y_4 = \frac{Y_7 F_{23}}{F_{24}} \quad (7.60)
\]

\[
X_5 = \frac{X_7 F_{24}}{F_{26}} \quad (7.61)
\]

\[
Y_5 = \frac{Y_7 F_{24}}{F_{26}} \quad (7.62)
\]

\[
X_5 = X_6 = X_4 \quad \text{(if } F_8 > 0 \text{)} \quad (7.63)
\]

\[
Y_5 = Y_6 = Y_4 \quad \text{(if } F_8 > 0 \text{)} \quad (7.64)
\]

\[
X_5 = Y_5 = 0 \quad \text{(if } F_8 < 0 \text{)} \quad (7.65)
\]

\[
X_6 = \frac{X_7 F_{26}}{F_{26} - F_8} \quad \text{(if } F_8 < 0 \text{)} \quad (7.66)
\]

\[
Y_6 = \frac{Y_7 F_{26}}{F_{26} - F_8} \quad \text{(if } F_8 < 0 \text{)} \quad (7.67)
\]

\[
X_7 = \frac{X_6 F_{10}}{2F_1 + F_{10}} \quad (7.68)
\]

\[
Y_7 = \frac{Y_6 F_{10} + 2F_1}{2F_1 + F_{10}} \quad (7.69)
\]
\[
X_i = \frac{X_i F_{13}}{F_3} \quad (7.70)
\]
\[
Y_i = \frac{Y_i F_{13}}{F_3} \quad (7.71)
\]
\[
X_i = \frac{2X_i + X_i}{3} \quad (7.72)
\]
\[
Y_i = \frac{2Y_i + Y_i}{3} \quad (7.73)
\]

By combining eqs. (7.57) to (7.73), both \(X_i\) and \(Y_i\) can be expressed as an explicit function of reaction extents [eq. (7.74) to (7.77)]. Therefore, all of the glucose and glutamine fractions can be determined from the material balance results.

\[
X_i = \frac{2F_3 + \frac{F_3 F_{14} F_{10}}{F_3 [F_{10} + 2 F_1]}}{3F_{26} - 2F_3 - \frac{F_{10} F_{14}}{F_{10} + 2 F_1}} \quad (if \ F_g > 0) \quad (7.74)
\]
\[
Y_i = \frac{2F_3 + \frac{F_3 F_{14} F_{26}}{F_3 [F_{10} + 2 F_1]}}{3F_{26} - 2F_3 - \frac{F_{10} F_{14}}{F_{10} + 2 F_1}} \quad (if \ F_g > 0) \quad (7.75)
\]
\[
X_i = \frac{2F_3 + \frac{F_3 F_{14} F_{10}}{F_3 [F_{10} + 2 F_1]}}{3[F_{26} - F_8] - 2F_3 - \frac{F_{10} F_{14}}{F_{10} + 2 F_1}} \quad (if \ F_g < 0) \quad (7.76)
\]
\[
Y_i = \frac{2F_3 + \frac{F_3 F_{14} [F_{26} - F_8]}{F_3 [F_{10} + 2 F_1]}}{3[F_{26} - F_8] - 2F_3 - \frac{F_{10} F_{14}}{F_{10} + 2 F_1}} \quad (if \ F_g < 0) \quad (7.77)
\]

The above analysis allows one to calculate the ATP production from each nutrient separately. Similar to the calculation of the total ATP production, the
following equations are derived from the reaction network. The ATP production from glucose and glutamine, defined by eqs. (7.78) and (7.79), respectively, is calculated according to the contributions of glucose and glutamine to each substrate. Since the catabolism of essential amino acids are assumed to be used only for energy production, the calculations as to their roles are greatly simplified. For example, the ATP production from leucine includes the ATP produced from the breakdown of leucine into acetyl-CoA and from the subsequent complete oxidation in the TCA cycle. According to the stoichiometry of the reaction network, the amount of ATP produced from the complete oxidation of acetyl-CoA is defined by eq. (7.80). As to threonine, valine, and isoleucine, their entry point to the TCA cycle is succinyl-CoA. One mole of succinyl-CoA yields five moles of NADH and two moles of GTP and FADH\(_2\) (Figure 7.6), respectively, as shown by eq. (7.81). The total ATP production from the catabolism of each amino acid is then defined by eqs. (7.82) to (7.86), respectively.

\[
\text{ATP}_{\text{gc}} = [2F_1 + Y_3 F_{24}] + [Y_7 F_{13} + Y_1 F_3 + Y_2 F_{23} + Y_4 F_{26}] \eta \text{N} + [2F_1 - Y_7 F_2 + Y_3 F_{26}] \eta \text{F} \tag{7.78}
\]

\[
\text{ATP}_{\text{gr}} = [X_3 F_{24} F_7] + [F_{5,5} + X_2 F_{23} + X_4 F_{26} + X_7 F_{13} + X_1 F_{23}] \eta \text{N} + [X_3 F_{5,5} X_2 F_{23}] \eta \text{F} \tag{7.79}
\]

\[
\text{ATP}^{\text{acsa}} = 1 + 3 \eta \text{N} + \eta \text{F} \tag{7.80}
\]

\[
\text{ATP}^{\text{suc}} = 2 + 5 \eta \text{N} + 2 \eta \text{F} \tag{7.81}
\]

\[
\text{ATP}_{\text{ac}} = F_{15} (3 \eta \text{N} + \eta \text{F} - 1 + \text{ATP}^{\text{acsa}} + \text{ATP}^{\text{suc}}) \tag{7.82}
\]

\[
\text{ATP}_{\text{ac}} = F_{15} (2 \eta \text{N} + \eta \text{F} - 2 + 3 \text{ATP}^{\text{acsa}}) \tag{7.83}
\]
Figure 7.6 Schematic diagram of the complete oxidation of succinyl-CoA through the TCA cycle
\[
\text{ATP}_\text{FAD} = F_1 \left( 5n_h + n_r + 2\text{ATP}^\text{sec} \right)
\]  
\[ (7.84) \]
\[
\text{ATP}_\text{ox} = F_2 \left( n_r - 1 + \text{ATP}^\text{sec} \right)
\]
\[ (7.85) \]
\[
\text{ATP}_\text{NAD} = F_2 \left( 4n_h + n_r - 1 + \text{ATP}^\text{sec} \right)
\]
\[ (7.86) \]

It is also possible to analyze the ATP production from different metabolic pathways. This information is useful for understanding the activity as well as the importance of each metabolic pathway. The amounts of ATP produced from the glycolytic pathway and the TCA cycle can be derived from the reactions listed in Table 7.1, and are defined by eqs. (7.87) and (7.88), respectively. The rest of the ATP is produced from catabolism of amino acids to form acetyl-CoA and succinyl-CoA, and is derived by subtracting the above two terms from the total, as shown by eq. (7.89).

\[
\text{ATP}_{\text{GLY}} = 2F_1 + (2F_1 - F_2) n_r
\]
\[ (7.87) \]
\[
\text{ATP}_{\text{TCA}} = F_2 + [F_3 + F_2 + F_2] n_r + F_2 n_r
\]
\[ (7.88) \]
\[
\text{ATP}_{\text{other}} = \text{ATP}_p - \text{ATP}_{\text{GLY}} - \text{ATP}_{\text{TCA}}
\]
\[ (7.89) \]

### 7.3.2 ATP Requirement

The free energy reserved in the phosphoanhydride bonds of ATP is required for the biosynthesis of cellular constituents. In the following calculations, the breakdown of one mole phosphate bonds is counted as the consumption of one mole ATP, since only one such bond is formed in the regeneration of ATP from
ADP. Hence, the breakdown of ATP to AMP in the synthesis of some nucleotides is counted as two ATPs.

In microbial systems, the ATP requirement for the synthesis of cellular components has been investigated for many years (Stouthamer, 1977). However, significant inconsistency can be found in the literature as to the biosynthetic ATP requirement. Also, detailed pathways for nucleotide synthesis were usually not considered. A more general and detailed approach is discussed below for the estimation of the biosynthetic ATP demand.

It should be emphasized that the definition of the ATP demand is ambiguous without specifying a common starting substrate for the synthesis of cellular components. For instance, the ATP requirement for lipid synthesis using glucose as substrate is significantly different from that using acetyl-CoA as the starting substrate (Figure 4.2). When the ATP production and demand are calculated, cautions need to be taken in order to avoid duplicate counting of the same reaction. In the following discussion, glucose and amino acids are assumed as the starting substrate for the biosynthesis of cellular components. The ATP production during the synthesis of cellular components is deducted from the total ATP requirement, and hence not included in the total ATP production. For instance, energy can be generated in the synthesis of lipids from glucose (Figure 4.2). This amount of energy is not included in the total ATP production, but rather it is deducted from the total ATP requirement. This approach provides a rational base for the calculation of the ATP demand and
production.

In protein synthesis, different numbers of ATP for the formation of each peptide bond were assumed in the literature (Paul, 1965; Stouthamer, 1977; Stouthamer, 1979a). A reasonable number should be 4 ATP/peptide bond. The activation of amino acid is coupled with the hydrolysis of one ATP into AMP, and hence this should be counted as two ATPs. The delivery of the activated amino acid (aminoacyl-tRNA) to the A site of the ribosome requires another ATP. No ATP is required for the formation of the peptide bond since sufficient free energy has already been reserved during the activation process. The translocation of the peptide chain from the A site to the P site requires another ATP. The total ATP demand for cellular protein and product formation is therefore four times of the total moles of the peptide bonds as defined by eq. (7.90).

\[
\text{ATP}_{\text{peptide}} = 4 \left( \frac{Z_p W}{M_p^{\text{op}}} + \frac{\theta_p}{M_p^{\text{pp}}} \right)
\]

(7.90)

The synthesis of carbohydrates is relatively simple if glycogen is assumed as the major carbohydrates. The incorporation of one glucose into polysaccharides requires two ATP, and therefore, the ATP demand for carbohydrate formation is calculated by eq. (7.91).

\[
\text{ATP}_{\text{carb}} = 2 F_{\text{Ia}}
\]

(7.91)

The ATP demand for lipid synthesis can be derived from the pathways shown in Figure 4.2. The net reducing power (NADPH) requirement is balanced through the productions from the pentose cycle and nucleotide synthesis. For the
addition of every four carbons (or the conversion of every glucose molecule) into fatty acids, four ATP molecules are consumed and two NADH molecules, one in the cytosol and one in the mitochondria, are produced. The cytosolic NADH is equivalent to one mitochondria FADH\textsubscript{2} in terms of the ATP production. Hence, the overall ATP requirement for lipid synthesis is then defined by eq. (7.92).

\[
ATP_{\text{lipid}} = (4 - \eta_N - \eta_F) F_{19}
\]  

(7.92)

The ATP demand for DNA and RNA synthesis includes the synthesis of nucleotides [eq. (4.4) to 4.11)] from the starting substrate, i.e., glucose and amino acids and the polymerization reactions. This approach is different from the method used in the literature (Paul, 1965; Stouthamer, 1977), in which detailed metabolic pathways for the synthesis of nucleotides are usually ignored. The polymerization reaction of DNA and RNA needs no additional ATP. The total ATP demands for DNA and RNA syntheses (including the ATP consumption for ribose-5-phosphate production from glucose) are defined by eqs. (7.93) and (7.94), respectively.

\[
ATP_{\text{DNA}} = N_{\text{DNA}} \left(7D_c + 8D_T + 8D_A + 9D_G - (D_c + D_T + D_A)\eta_N\right) + N_{\text{DNA}}
\]  

(7.93)

\[
ATP_{\text{RNA}} = N_{\text{RNA}} \left(7R_c + 6R_u + 8R_A + 9R_G - (R_c + R_u + R_A)\eta_N\right) + N_{\text{RNA}}
\]  

(7.94)

Active transport of nutrients into cells also requires energy. Unfortunately, the exact mechanism of nutrient transport in animal cells is not well understood yet. Hence, it is assumed that one mole ATP is required for the transport of one mole nutrient. It should be mentioned that the transport of nutrients required for energy production is not included. The ATP demands for transport of glucose
and amino acids are then derived from the amounts of glucose and amino acids required for the synthesis of cell mass and product, and are defined by eqs. (7.95) and (7.96), respectively. The ATP demand for the transport of glucose and amino acids used for energy production is not included because the amount of nutrients used for this purpose is not constant and is dependent on the nutritional environment. The total stoichiometric ATP demand for the transport of biosynthetic substrates is then calculated from eq. (7.97).

\[ \text{ATP}_{sc} = F_{17} + F_{18} + F_{19} + F_{20} \]  
(7.95)

\[ \text{ATP}_{AA} = \frac{Z_\text{p}W}{M_p} + \frac{\theta_2}{M_p} \]  
(7.96)

\[ \text{ATP}_{sc} = \text{ATP}_{sc} + \text{ATP}_{AA} \]  
(7.97)

The overall stoichiometric ATP demand for the synthesis of cell mass and product (including transport) is then the total of the above and is defined by eq. (7.98).

\[ \text{ATP}_{\text{int}} = \text{ATP}_{\text{peptid}} + \text{ATP}_{\text{carb}} + \text{ATP}_{\text{lipid}} + \text{ATP}_{\text{DNA}} + \text{ATP}_{\text{RNA}} + \text{ATP}_{\text{sc}} \]  
(7.98)

### 7.4 Energy Balance Results and Discussions

#### 7.4.1 Effective P/O Ratio

The mitochondrial oxidative phosphorylation of NADH and FADH$_2$ involves three coupling sites: NADH-Q reductase (site 1), cytochrome reductase (site 2), and cytochrome oxidase (site 3) (Hinkle et al., 1979). Electrons from NADH are transferred to O$_2$ through sites 1, 2, and 3, while electrons from FADH$_2$ enter the chain at site 2. The use of protons to drive ATP/ADP$+P_i$ transport lowers the
traditional P/O ratio of 1 per coupling site (Hinkle et al., 1991). The mechanistic (or maximum) P/O ratios at the individual coupling sites have been determined by Stoner (1987) with bovine heart mitochondria and by Hinkle et al. (1991) with rat liver mitochondria to be 1.0 at sites 1 and 3, and 0.5 at site 2. This results in mechanistic P/O ratios of 2.5 for NADH and 1.5 for FADH$_2$. In resting hepatocytes, the effective P/O ratio was found to be as low as 50% of the mechanistic P/O ratio due to proton leak (25-30%) and heat production (15-20%) (Brand et al., 1993; Nobes et al., 1990). However, in actively dividing cells, the proton leak is expected to be quantitatively less important as shown by Nobes (1990). Hence, the effective P/O ratio in hybridoma cell culture is estimated to be 80% of the mechanistic P/O ratio, which results in effective P/O ratios of 2.0 for NADH and 1.2 for FADH$_2$.

7.4.2 Analysis of ATP Production

The stoichiometric ATP production determined from the four different fed-batch and batch cultures are shown in Table 7.7. The average specific cell growth rate is calculated by dividing the total number of cells produced over the concerned time period by the time integral of viable cells. The direct ATP production without the participation of oxidative phosphorylation accounts for a significant portion of the total. For example, in the fed-batch cultures, about 20% of the total ATP is produced directly, while the percentage increases to 41% in the batch culture. Apparently, the difference is caused by the decrease in glucose consumption through the glycolytic pathway in the fed-batch cultures. The
Table 7.7 Stoichiometric ATP production from fed-batch and batch cultures of hybridoma CRL-1606 cells.

<table>
<thead>
<tr>
<th></th>
<th>FB1</th>
<th>FB2</th>
<th>FB3</th>
<th>B4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*</td>
<td>%</td>
<td>*</td>
<td>%</td>
</tr>
<tr>
<td>Total ATP Production</td>
<td>37.2</td>
<td>40.6</td>
<td>34.7</td>
<td>34.7</td>
</tr>
<tr>
<td>Direct ATP &amp; GTP</td>
<td>8.6</td>
<td>23.0</td>
<td>8.8</td>
<td>21.7</td>
</tr>
<tr>
<td>From NADH</td>
<td>22.4</td>
<td>60.1</td>
<td>25.0</td>
<td>61.4</td>
</tr>
<tr>
<td>From FADH$_2$</td>
<td>6.3</td>
<td>16.9</td>
<td>6.8</td>
<td>16.9</td>
</tr>
</tbody>
</table>

* unit: mmole/10$^9$ cell
oxidative phosphorylation of NADH contributes to more than 60% of the ATP production in the fed-batch cultures and about 47% in the batch culture. The production of ATP from the oxidative phosphorylation of FADH$_2$ contributes to only 12 to 19% of the total ATP production.

The ATP production from different metabolic pathways in the four culture experiments are listed in Table 7.8. Not surprisingly, a significant fraction (41%) of the ATP in the batch culture is formed from the anaerobic metabolism of glucose through the glycolytic pathway, as reported by many others (Bonarius et al., 1996; Glacken et al., 1986). However, when glucose is well controlled, this fraction decreases to 22% in one of the fed-batch cultures. In all cases, the TCA cycle provides majority of the ATP requirement (51 - 68%). This suggests that the essential role of the TCA cycle cannot be replaced by the glycolytic pathway, and that the TCA cycle is quite active even at a high glucose concentration. The amount of ATP produced from the catabolism of amino acids to form acetyl-CoA and succinyl-CoA accounts for about 10% of the total ATP requirement.

7.4.3 Contribution of Nutrients to ATP Production

The contributions of glucose and glutamine to the TCA cycle intermediates and to the production of lactate and non-essential amino acids are calculated using the fractional contribution model. The results obtained from the four experiments are presented in Table 7.9. With this information, the contributions of glucose and glutamine to the total ATP production can be readily calculated. The
Table 7.8 Stoichiometric ATP production from different pathways in fed-batch and batch cultures of hybridoma CRL-1606 cells.

<table>
<thead>
<tr>
<th></th>
<th>FB1</th>
<th>FB2</th>
<th>FB3</th>
<th>B4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*</td>
<td>%</td>
<td>*</td>
<td>%</td>
</tr>
<tr>
<td>Total ATP Production</td>
<td>37.2</td>
<td>40.6</td>
<td>34.7</td>
<td>34.7</td>
</tr>
<tr>
<td>From glycolysis</td>
<td>10.1</td>
<td>27.2</td>
<td>10.3</td>
<td>25.3</td>
</tr>
<tr>
<td>From TCA Cycle</td>
<td>23.4</td>
<td>62.9</td>
<td>26.0</td>
<td>64.0</td>
</tr>
<tr>
<td>From Others</td>
<td>3.7</td>
<td>9.9</td>
<td>4.4</td>
<td>10.7</td>
</tr>
</tbody>
</table>

* unit: mmole/10^9 cell

Table 7.9 Calculated molar fractional contributions of glutamine and glucose to each reaction labeled in Figure 7.5

<table>
<thead>
<tr>
<th>Subscript</th>
<th>FB1</th>
<th>FB2</th>
<th>FB3</th>
<th>B4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X_1</td>
<td>Y_1</td>
<td>X_1</td>
<td>Y_1</td>
</tr>
<tr>
<td>1</td>
<td>0.268</td>
<td>0.666</td>
<td>0.314</td>
<td>0.647</td>
</tr>
<tr>
<td>2</td>
<td>0.396</td>
<td>0.550</td>
<td>0.456</td>
<td>0.512</td>
</tr>
<tr>
<td>3</td>
<td>0.393</td>
<td>0.546</td>
<td>0.453</td>
<td>0.509</td>
</tr>
<tr>
<td>4</td>
<td>0.390</td>
<td>0.541</td>
<td>0.450</td>
<td>0.505</td>
</tr>
<tr>
<td>5</td>
<td>0.000</td>
<td>0.000</td>
<td>0.450</td>
<td>0.505</td>
</tr>
<tr>
<td>6</td>
<td>0.384</td>
<td>0.533</td>
<td>0.450</td>
<td>0.505</td>
</tr>
<tr>
<td>7</td>
<td>0.037</td>
<td>0.955</td>
<td>0.043</td>
<td>0.953</td>
</tr>
<tr>
<td>8</td>
<td>0.036</td>
<td>0.932</td>
<td>0.042</td>
<td>0.930</td>
</tr>
</tbody>
</table>
contributions of essential amino acids to the ATP production can also be estimated by assuming that their carbon skeletons are completely oxidized in the TCA cycle. Table 7.10 shows the contribution of each nutrient to the ATP production. In all cases, the total amount of ATP derived from the complete oxidation of essential amino acids is insignificant (ranges from 3 to 11%). In FB3 where both glucose and glutamine were controlled at very low levels, the percentage of ATP derived from glutamine is the lowest (18%). Even in the batch culture where both glucose and glutamine were in excess, the amount of ATP produced from glutamine is less than 30%. In all four cases, glucose is the major source for the ATP production.

### 7.4.4 Respiratory Quotient

Oxygen is the terminal acceptor for the electrons transferred from NADH and FADH₂. The stoichiometry shows that the oxidation of one mole of NADH or FADH₂ requires 0.5 mole of oxygen. Accordingly, the stoichiometric oxygen requirement is calculated from the production of NADH and FADH₂ as shown by eq. (7.99). The CO₂ production can be derived according to the reactions of the metabolic network. The respiratory quotient (RQ), defined as the ratio of carbon dioxide production over the oxygen consumption, is then calculated from eq. (7.101).

\[
\theta_{O_2} = \frac{\text{ATP}^N}{2\eta_n} + \frac{\text{ATP}^F}{2\eta_f}
\]  
(7.99)

\[
\theta_{\text{CO}_2} = F_7 + F_{10} + F_{13} + 2F_{14} + F_{17} + 2F_{19} + 6F_{20} + F_{22} + F_{33} - N_N - N_G
\]  
(7.100)
Table 7.10 Contributions of nutrients to ATP production in hybridoma batch and fed-batch cultures.

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>FB1 %</th>
<th>FB2 %</th>
<th>FB3 %</th>
<th>B4 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>72.9</td>
<td>69.0</td>
<td>75.9</td>
<td>60.2</td>
</tr>
<tr>
<td>Glutamine</td>
<td>24.0</td>
<td>27.8</td>
<td>17.8</td>
<td>28.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.2</td>
<td>0.9</td>
<td>1.9</td>
<td>7.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.0</td>
<td>0.3</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.2</td>
<td>1.4</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Valine</td>
<td>0.0</td>
<td>0.6</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>
\[ RQ = \frac{\theta_{\text{CO}_2}}{\theta_{\text{O}_2}} \] (7.101)

The above parameters are determined using the material balance data obtained from the four experiments, and the results are presented in Table 7.11. The calculated stoichiometric oxygen consumption differs significantly among the fed-batch and batch cultures. This is probably because in the batch culture, a significant amount of ATP is derived from the anaerobic oxidation of glucose via the glycolytic pathway due to the significant high lactate production. It should be emphasized that the dissolved oxygen concentrations in FB3 and B4 were controlled at the same level. Hence, the difference is not caused by the dissolved oxygen level. Apparently, cells choose to use glucose less efficiently when glucose is in excess. The oxygen uptake rate (OUR) can be expressed as a function of cell growth and the stoichiometric oxygen consumption, as shown by eq. (7.102). Obviously, the estimated OUR is not a linear function of cell growth since the stoichiometric oxygen consumption changes with the culture conditions, especially with glucose concentrations. Results also show that \( \mu \theta_{\text{O}_2} \) is not constant, which suggests that OUR may not be a linear function of viable cell density \( X_v \) either. On the other hand, the respiratory quotient is relatively constant (ranges from 1.18 to 1.29). It should be noted that the above discussions are based on the stoichiometric oxygen consumption calculated using the model. No oxygen consumption or carbon dioxide production was measured in those experiments. However, Zupke and Stephanopoulos (1995a) measured the specific oxygen consumption rate as 2.0 mmole/10^{10} cells-hr, and
Table 7.11 Oxygen consumption and carbon dioxide production in hybridoma batch and fed-batch cultures.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>FB1</th>
<th>FB2</th>
<th>FB3</th>
<th>B4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\theta_{o_2}$ (mmole/10^9 cells)</td>
<td>8.4</td>
<td>9.3</td>
<td>8.6</td>
<td>5.7</td>
</tr>
<tr>
<td>$\theta_{o_2} \mu$ (mmole/10^9 cells-h)</td>
<td>0.14</td>
<td>0.11</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>$\theta_{co_2}$ (mmole/10^9 cells)</td>
<td>10.3</td>
<td>10.9</td>
<td>10.2</td>
<td>7.4</td>
</tr>
<tr>
<td>RQ</td>
<td>1.22</td>
<td>1.18</td>
<td>1.19</td>
<td>1.29</td>
</tr>
</tbody>
</table>
the production rate of carbon dioxide as 2.6 mmole/10^{10} cells-h, in a batch culture with the same cell line used in this work (hybridoma CRL-1606). The specific growth rate was measured in the same batch culture (Zupke, 1993) as 0.033/h. The stoichiometric oxygen consumption, CO_2 production, and the respiratory quotient can then be calculated from these experimentally measured data to be 6.1 mmole/10^9 cells, 7.9 mmole/10^9 cells, and 1.3, respectively. The stoichiometric oxygen consumption (5.7 mmole/10^9 cells), CO_2 production (7.4 mmole/10^9 cells), and the respiratory quotient (1.29) calculated using the model for the batch culture (B4) (see Table 7.11), are strikingly close to the measured values in the batch culture by Zupke (1993). These results support that the model developed in this work is valid and reliable.

\[
\text{OUR} = \theta_0 \frac{dX}{dt} = \theta_0 \mu X, \tag{7.102}
\]

### 7.4.5 ATP Yield and Maintenance Demand

According to the maintenance model, the specific ATP consumption rate can be expressed as a function of growth rate as shown by eq. (7.103). This assumes that there are two terms of ATP requirement: a growth associated term \(m_{\text{ATP}}\) and a non-growth associated term \(1/Y_{\text{ATP}}^{\text{max}}\). By assuming that no ATP accumulates in the cells, the total ATP consumption should then equal the total ATP production. According to the definition, \(q_{\text{ATP}}\) can be calculated by multiplying the stoichiometric ATP production with the specific cell growth rate, as described by eq. (7.104).

\[
q_{\text{ATP}} = \frac{\mu}{Y_{\text{ATP}}^{\text{max}}} + m_{\text{ATP}} \tag{7.103}
\]
\[ q_{\text{ATP}} = \mu \times \text{ATP}^\text{ext} \]

(7.104)

In order to determine \( Y_{\text{ATP}}^{\text{max}} \) and \( m_{\text{ATP}} \), the specific ATP consumption rates from the four experiments are calculated. The culture span of FB3 is divided into three phases: an exponential phase, a stationary phase, and a death phase. Each phase is treated as a separate experiment. The specific ATP consumption rate is also calculated for each phase, and the results are shown in Table 7.12. A linear relationship between the specific ATP consumption rate and the specific growth rate is shown in Figure 7.7. The slope of the straight line is the reciprocal of the theoretical ATP yield and the intercept is the maintenance ATP demand. According to the slope of the line, \( Y_{\text{ATP}}^{\text{max}} \) is found to be \( 3.5 \times 10^7 \) cells/m mole ATP, which corresponds to \( 8.7 \) mg cells/m mole ATP in accordance with the dry cell weight of \( 25 \) mg /\( 10^8 \) cells. This value is comparable to the yield commonly found in microbial systems (Bauchop and Elsden, 1960; Southamer, 1977). The maintenance ATP demand is found from the intercept, to be \( 0.14 \) mmole ATP/\( 10^9 \) cells-h.

The ATP demand for the synthesis of cell mass and product can be estimated on the basis of cell composition and dry cell weight. The results are listed in Table 7.13. The total ATP requirement for the synthesis of cell mass and product (not including transport of nutrients) is estimated to be \( 8.52 \) mmole ATP/\( 10^9 \) cells. The ATP demand for the synthesis of the cellular proteins and product accounts for about 90% of the total. According to the above determined \( Y_{\text{ATP}}^{\text{max}} \), the non-growth associated ATP demand is \( 28.6 \) mmole ATP/\( 10^9 \) cells. The ATP demand for the transport of extracellular nutrients is then assumed to be part of...
Table 7.12. The stoichiometric ATP production and the specific ATP consumption rate in hybridoma batch and fed-batch cultures.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time Period h</th>
<th>μ 1/h</th>
<th>ATPₚₐ mmole/10⁶ cells</th>
<th>qₐₚ mmole/10⁶ cells-h</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1</td>
<td>0 - 349</td>
<td>0.016</td>
<td>37.2</td>
<td>0.61</td>
</tr>
<tr>
<td>FB2</td>
<td>0 - 334</td>
<td>0.012</td>
<td>40.6</td>
<td>0.48</td>
</tr>
<tr>
<td>FB3</td>
<td>0 - 104</td>
<td>0.023</td>
<td>34.7</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>104 - 213</td>
<td>0.022</td>
<td>33.4</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>213 - 285</td>
<td>0.017</td>
<td>30.1</td>
<td>0.79</td>
</tr>
<tr>
<td>B4</td>
<td>0 - 91</td>
<td>0.033</td>
<td>35.0</td>
<td>1.16</td>
</tr>
</tbody>
</table>
Figure 7.7 Specific ATP production rate as a function of the specific cell growth rate in hybridoma cell cultures.
Table 7.13 Theoretical maximum ATP demand for cell mass and product formation.

<table>
<thead>
<tr>
<th></th>
<th>ATP Demand mmole ATP/10^8 cells</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biosynthetic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular Proteins</td>
<td>6.37</td>
<td>74.8</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>0.10</td>
<td>1.1</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.43</td>
<td>5.0</td>
</tr>
<tr>
<td>RNA</td>
<td>0.18</td>
<td>2.2</td>
</tr>
<tr>
<td>DNA</td>
<td>0.07</td>
<td>0.8</td>
</tr>
<tr>
<td>Product</td>
<td>1.36</td>
<td>16.0</td>
</tr>
<tr>
<td><strong>Transport</strong></td>
<td>2.63</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Amino Acids</td>
<td>1.93</td>
<td></td>
</tr>
</tbody>
</table>
the requirement for biosynthesis. The total ATP demand for the active transport of glucose (only the fraction needed for cell mass and product synthesis) and amino acids is estimated to be 2.61 mmole ATP/10⁶ cells. Addition of the above two values then gives 11.13 mmole ATP/10⁶ cells. The above results suggest that the biosynthetic ATP demand could be significantly underestimated, and/or the non-growth associated ATP demand may contain some ATP requirements for purposes other than cell mass and product synthesis.

7.5 Sensitivity Analysis

The material and energy balances were performed using experimental measurements with certain errors. It is therefore necessary to evaluate the sensitivity of the mathematical model with respect to changes of certain parameters. Fed-batch culture FB3 was chosen in the following sensitivity analysis. Glucose or glutamine consumption was varied by ±10% of its measured value while all other parameters were kept unchanged. It should be mentioned that the cumulative nutrient consumption in a fed-batch culture is dominated by the amount of nutrient fed from the supplemental medium, and hence is relatively insensitive to the errors in the determination of nutrient concentration in the culture medium.

7.5.1 Sensitivity of RQ to Glucose and Glutamine Consumption

A 10% increase in the total glucose consumption results in a 15% increase in oxygen consumption, a 13% increase in CO₂ production, and a 3% increase in the respiratory quotient. A 10% decrease leads to a 20% decrease in oxygen
consumption, a 13% decrease in CO₂ production, and a 3% decrease in the respiratory quotient. These results indicate that the oxygen consumption and CO₂ production are sensitive to the glucose consumption but the respiratory quotient is not.

A 10% increase in the total glutamine consumption results in a 3% increase in oxygen consumption, a 3% increase in CO₂ production, and a 1% increase in the respiratory quotient. A 10% decrease results in a 4% decrease in oxygen consumption, a 4% decrease in CO₂ production, and a 0.3% decrease in the respiratory quotient. These results show that the oxygen consumption, CO₂ production, and respiratory quotient are insensitive to the glutamine consumption.

7.5.2 Sensitivity of ATP Production to Glucose and Glutamine Consumption

As expected, ATP production is very sensitive to the total glucose consumption. This is shown by the following facts: a 10% increase in the total glucose consumption leads to an 18% increase in the total ATP production, and a 10% decrease in the total glucose consumption results in a 13% decrease in the total ATP production. Because the stoichiometric demand for cell mass synthesis is fixed and the lactate production is also unchanged, changes in the total glucose consumption reflect the change of glucose metabolized in the TCA cycle.

The ATP production is less sensitive to the total glutamine consumption as shown by the 7% increase in the ATP production resulted from a 10% increase
in the total glutamine consumption. This is because glutamine provides less than 20% of the total ATP production.

7.5.3 Sensitivity to Amino Acid Composition

Two amino acids, leucine (essential) and aspartate (non-essential), were chosen in the analysis of the sensitivity of the model to amino acid composition changes. In the following analysis, the leucine composition of cellular proteins was increased from 8.1% to 10.5% (a change of 30%) while the aspartate composition was decreased from 4.7% to 2.3% (a change of 51%). Consequently, the percentage difference between the stoichiometric leucine consumption and calculated demand for protein synthesis changes from 20.7% (Table 7.4) to -6.7%. The amount of aspartate entering the TCA cycle changes from 0.002 mmole/10⁹ cells to 0.047 mmole/10⁹ cells. This change has no effect on the total synthesis of NAAs from glutamine, and hence no effect on the glutamine nitrogen balances. The total ATP production is reduced by 3% as a result of the amino acid composition changes. No effects on the oxygen consumption, CO₂ production, and the respiratory quotient is observed. The above results suggest that the model is insensitive to the changes of the amino acid composition of cellular proteins. Because the product accounts for less than 20% of the total protein synthesis (including cellular proteins and the product), changes in the amino acid composition of the product failed to show any significant effect on the material or energy balances.
8. CHO Cell Cultures

Three CHO cell lines were chosen to examine the applicability of the integrated approach for medium design and nutritional control to different culture systems. The development of a serum-free medium and the implementation of the integrated approach in fed-batch cultures with the CHO cell lines were completed in three months. Results obtained with one of the three CHO cell lines are discussed in detail below.

8.1 CHO Cell Growth

One fed-batch culture and one batch culture were carried out in spinner flasks with CHO cells producing interferon-γ. The fed-batch culture was initiated with Init-D (see Chapter 3), and the batch with Init-D supplemented with 20 mM glucose and 4 mM glutamine. Cell growth profiles are shown in Figure 8.1. The maximum viable cell density in the fed-batch culture was increased to $5 \times 10^6$ cells/ml as compared to $2.4 \times 10^6$ cells/ml in the batch culture. The culture span was also increased to 200 hours as a result of the stoichiometric feeding. Unlike the hybridoma cultures where cell growth rate was maintained relatively constant, the specific growth rate in the CHO cultures decreased dramatically in the stationary growth phase. The exact cause needs to be investigated further.
Figure 8.1 Comparison of viable cell density in batch and fed-batch cultures of CHO cells in spinner flasks.
8.2 Nutritional Control and By-product Formation

Glucose was controlled at very low concentration in the fed-batch culture as shown in Figure 8.2. Similar to the hybridoma cultures, lactate production was significantly reduced in the fed-batch culture. This is shown by the six fold reduction in the specific lactate production rate (from 0.15 mmole/10^9 cells-h in batch to 0.024 mmole/10^9 cells-h in the fed-batch). Although the maximum lactate concentration was less than 25 mM and hence probably not detrimental to the cell growth, further reduction is necessary to significantly increase the viable cell density. It should be noted that manual feeding was employed which led to instantaneous increases in glucose concentration during the culture process. It is therefore expected that automatic feeding and process control can solve the problem with lactate accumulation. The glutamine concentration and ammonia production in the fed-batch culture are shown in Figure 8.3. Glutamine reached a maximum concentration of 6.3 mM in the fed-batch culture. The glutamine accumulation was partially caused by the overfeeding resulted from the overestimation of the cell growth rate which decreased significantly at the stationary phase. It is also possible that the glutamine concentration in the supplemental medium was overestimated. It should be emphasized that the initial medium contains 2 g/L Primatone RL, a meat hydrolysate that is equivalent to about 18 mM of total amino acids. The total amount of amino acids in the initial medium is therefore estimated to be 27 mM as compared to 5.5 mM in the Init-B (see Chapter 3). The excessive amount of amino acids in the initial medium could inhibit the biosynthesis of non-essential
Figure 8.2 Glucose concentration and lactate production in fed-batch and batch cultures of CHO-γ cells with spinner flasks
Figure 8.3 Glutamine concentration and ammonia production in a fed-batch culture of CHO cells with spinner flask.
amino acids from glutamine which leads to a decrease in glutamine consumption. However, the specific ammonia production rate was reduced only slightly from 0.025 mmole/10^9 cells-h in the batch to 0.016 mmole/10^9 cells-h in the fed-batch culture. The excessive amount of amino acids could also directly contribute to the ammonia production as shown in the material balances of hybridoma cultures (see Chapter 7). Both essential and non-essential amino acids can be metabolized for energy production while the amino group can be released into free ammonia. Obviously, the initial medium is far from optimized. Concentration of Primatone RL needs to be reduced. The amount of amino acids contained in the Primatone RL should be considered in the design of the initial medium. It is possible that no free amino acids need to be included in the initial medium. Reduction of ammonia formation through the initial medium optimization and automatic feeding is necessary to improve the culture performance further.

8.3 Product Formation

The concentrations of interferon-γ in the batch and fed-batch cultures are shown in Figure 8.4. Because of the increased viable cell density and extended culture life, the maximum concentration of interferon-γ was increased from 7.6 mg/L in the batch to 30 mg/L in the fed-batch culture. When the total interferon-γ production was plotted against the time integral of the viable cells, a linear correlation was obtained as shown in Figure 8.5, indicating that the production rate was constant. The specific interferon-γ production rate can be calculated
Figure 8.4 Concentration of interferon-\(\gamma\) in batch and fed-batch cultures of CHO cells with spinner flasks.
Figure 8.5 Specific interferon-γ production rate in batch and fed-batch cultures of CHO cells with spinner flasks.
from the slope of the line, and was found to be essentially the same in the batch and fed-batch cultures. Hence, the increase in the interferon-γ concentration in the fed-batch culture was solely due to the increased culture viability.

8.4 Applications to Other CHO Cells

The same serum-free medium (Init-D, see Chapter 3) was employed in the cultivation of two other CHO cell lines (denoted as CHO-a and CHO-b) obtained from industry. Fed-batch cultures were conducted in 500 ml spinner flasks using the same supplemental medium (Sup-IV, see Chapter 3) as in the cultures of CHO cells producing interferon-γ. Two batch cultures were also conducted with the cell line CHO-a. One, denoted as Batch-A, was initiated with medium Init-D supplemented with 4 mM glutamine and 20 mM glucose in a 150 ml spinner flask. The other, denoted as Batch-B, was initiated with JRH protein-free medium supplemented with 2 g/L Primatone RL, 2 mg/L insulin, and 2 mg/L transferrin in a 250 ml shake flask. The viable cell densities from the batch and fed-batch cultures with cell line CHO-a are shown in Figure 8.6. The maximum viable cell density in the fed-batch was increased by 2- and 3-fold as compared to those in the Batch-A and Batch-B cultures, respectively. The integrated viable cell density with time was increased by 3- and 4-fold, respectively. As a result, the final product titer was increased by 2.4- and 2.6-fold, respectively. A dramatic decrease in the specific product synthesis rate was observed from the growth phase to the stationary phase. Differences in the specific product synthesis rate were also found in the different cultures. One possible
Figure 8.6 Comparison of viable cell density in batch and fed-batch cultures with an industrial CHO cell line.
explanation for this behavior is that the cell line is unstable under the culture
conditions investigated, especially when methotrexate is not added to the
culture medium. This is supported by the fact that the productivity decreased
after a few passages of growth without methotrexate. It is also possible that the
change in productivity was associated with cell growth since a significant
decrease in cell growth rate was observed in the stationary phase. This
phenomenon needs further investigation.

Significant improvement in maximum cell density and product formation was
also achieved in the fed-batch culture with the cell line CHO-b. The maximum
viable cell density was increased by 3-fold as compared to a typical batch
culture, and the final product titer by 3.5-fold.

The improvement obtained in fed-batch cultures with three different 
CHO cell
lines suggests that the integrated approach for medium design and nutritional
control is applicable to different systems. The fact that a very short period of time
was devoted to the development of the supplemental medium indicates that the
approach is more rapid and effective than empirical approaches.
9. Conclusions

This thesis was focused on two critical issues: the quantification of the nutritional demands for animal cell growth and the control of the nutritional environment for the reduction of toxic by-product formation. The conclusions are summarized below.

Detailed analysis of the effects of key nutrients (glucose and glutamine) on the kinetics of cell growth and by-product formation led to the design of an optimal nutritional environment to minimize by-product formation while maintaining a reasonable cell growth rate. The optimal concentrations of glucose and glutamine were found to be twice their $K_m$ values in the cell growth kinetics. It is concluded that commercial media are not optimal for high density cultures.

The metabolic reaction network was studied in detail to derive the overall stoichiometric equation governing animal cell growth. The biosynthesis of non-essential amino acids from glutamine was carefully investigated. It was hypothesized that maximum uptake of non-essential amino acids from culture medium led to a minimum glutamine consumption and hence minimized ammonia production. Choosing the maximum uptake of non-essential amino acids and a minimum lactate to glucose ratio as design parameters to minimize by-product formation allows the determination of the stoichiometric equation as a unique function of cell composition and energy demand. The stoichiometric equation defines the minimal nutritional demands for animal cell growth. The stoichiometric coefficients were applied in the design of stoichiometrically
balanced supplemental media for feeding. Feeding strategy was also developed to balance the feed with the demand for each individual nutrient required for cell growth.

Fed-batch hybridoma cultures were conducted in T-flasks and a 2-liter bioreactor with the feeding of the stoichiometrically balanced supplemental media and the fed-on-demand strategy for nutritional control. The nutritional environment (judged by the concentrations of glucose, glutamine, and essential amino acids) was controlled relatively constant in the exponential growth phase. By-product formation (lactate, ammonia, and non-essential amino acids) was significantly reduced as a result of the nutritional control. Very high viable cell densities were achieved which led to a 50-fold increase in the final product concentration (2400 mg/L) and a 10-fold increase in the volumetric productivity as compared to a batch culture. The specific product synthesis rate was found to be constant over the fed-batch culture process and equal in different cultures.

Extracellular measurements from batch and fed-batch hybridoma cultures were employed in material and energy balances to explore insights into intracellular metabolism. The measured stoichiometric consumption of most essential amino acids was found to be about the same as the calculated demands for protein synthesis, indicating the essential roles of essential amino acids are to provide precursors for protein synthesis. Analysis of the metabolic fates of glucose and glutamine revealed that the metabolism of key nutrients is strictly regulated under different conditions to ensure sufficient energy production for cell growth. A positive relationship among the ammonia production, non-essential amino
acid biosynthesis, and the glutamine consumption was observed. This confirms the hypothesis that maximum uptake of non-essential amino acids from culture medium leads to minimum ammonia production.

Applications of the stoichiometric approach in medium design and nutritional control to three different CHO cell lines resulted in significant improvement in culture performance in a very short period of time. These results indicate that the integrated approach is effective and valid to different culture systems.
10. Recommendations

10.1 Cell Death

With stoichiometric feeding and nutritional control, cell growth has been successfully extended to achieve a maximum product titer. Cell death event was delayed for a certain period of time but was not successfully avoided. Successful knockout of the apoptosis pathway will certainly allow a wider window for process control, but without eliminating the factors that cause cell death, it is unlikely that cell death can be completely prevented. This is because necrosis becomes dominant when the environment is reversed. It is therefore necessary to investigate the metabolic events that lead to cell death. From the fed-batch hybridoma cultures, it is evident that cell death is related with altered metabolic behavior. In the stationary phase of cell culture, lactate was consumed instead of being produced as in the growth phase. The cause for the change of the direction of the pyruvate conversion to lactate is not very clear. This change leads to a net production instead of consumption of cytosolic NADH. Understanding the effects of these changes on cell metabolism may reveal the factors that are responsible for cell death. In addition to the changes in the cytosol, changes in the mitochondria were also observed when cell death occurred. In the stationary phase, the total synthesis of serine, alanine, and aspartate increased significantly and exceeded the amount of glutamate transaminated. This indicates that a significant amount of free ammonia was incorporated into NAA through the reverse reaction of the glutamate hydrolysis. Although the net conversion of glutamate to α-ketoglutarate was positive, a
significant amount of mitochondrial NADH was consumed. This may or may not be the direct cause for cell death, but understanding the mechanism involved in the change and its impact on energy metabolism will certainly be critical to solve the problem. Based upon the above analysis, it is recommended that the effects of the environmental changes on the intracellular metabolic reaction extents/fluxes be carefully studied to identify the factors that are responsible for cell death.

10.2 Serum-free Defined Media

This thesis was focused on the nutritional demands for glucose, amino acids, and vitamins for cell growth. Empirical approaches were taken to develop the serum-free medium and to feed serum and trace metals to the hybridoma fed-batch cultures. Due to concerns with possible virus contamination, serum-free media will be required for the manufacturing of therapeutic proteins for human diseases. However, the substances in the animal sera that are essential for cell growth have not been identified. Current serum-free media employed for research or production contain undefined components such as protein hydrolysates. The development of these media was empirical without the knowledge of the quantitative demands of these components for cell growth. Consequently, maximum cell densities achievable under serum-free conditions are usually very low. The understanding of the complicated nutritional demands including growth promoting factors, trace elements, and protecting reagents is hence indispensable for the optimization of cell culture processes using completely defined media.
10.3 Applications of Medium design in Continuous and Perfusion Cultures

One of the advantages of continuous and perfusion cultures is the high volumetric productivity. However, both processes suffer from a common disadvantages: low product concentration. This is because high dilution rate is essential to maintain cells viable by providing sufficient nutrients and diluting the toxic by-product concentrations below their detrimental levels. The problem comes from the medium which contains very low concentrations of the nutrients required for the synthesis of cell mass. The stoichiometric approach devised in this thesis allows the design of a balanced medium with high concentrations of the most consumed nutrients to lower the dilution rate in perfusion culture and to increase the viable cell density in continuous culture. High product concentration is expected, which will dramatically reduce medium usage, storage tank size, and purification load. However, a balanced salt concentration and proper osmolality need to be maintained, which requires careful simulation of the culture process and design of the culture medium.

10.4 Protein Quality

The integrity of the protein backbone and the attachment and the structure of sugar chains are vital to the proper biological functions and stability of therapeutic proteins. Preliminary studies (data not shown) indicate that glucose starvation leads to a low frequency in glycosylation. On the other hand, low
glucose and glutamine concentrations are favorable for the reduction of lactate and ammonia formation. Therefore, there exists an optimal condition where by-product formation is reduced without sacrificing the protein quality. Further studies on the effects of key nutrient concentrations on protein quality are necessary. It will also be useful to investigate protein quality under a controlled nutritional environment using the approach devised in this thesis to achieve consistency and reproducibility in product titer and quality.
Nomenclature

\( A_i \)  \( \) total consumption of nutrient i (all 20 amino acids), mmole

\( \text{ATP}^{\text{ace}}, \text{ATP}^{\text{suc}} \)  \( \) moles of ATP produced from the complete oxidation of one mole acetyl-CoA and succinyl-CoA, respectively, mmole ATP/mmole substrate

\( \text{ATP}_{\text{gic}}, \text{ATP}_{\text{gin}} \)  \( \) stoichiometric ATP production from glucose and glutamine, respectively, mmole ATP/cell

\( \text{ATP}_{\text{GLY}}, \text{ATP}_{\text{TCA}} \)  \( \) stoichiometric ATP production from the glycolytic pathway and in the TCA cycle, respectively, mmole ATP/cell

\( \text{ATP}_{\text{ile}}, \text{ATP}_{\text{leu}}, \text{ATP}_{\text{lys}} \)  \( \) stoichiometric ATP production from isoleucine, leucine, and lysine, respectively, mmole ATP/cell

\( \text{ATP}_{\text{other}} \)  \( \) stoichiometric ATP production from pathways other than the glycolytic pathway and in the TCA cycle, mmole ATP/cell

\( \text{ATP}_{\text{Carb}}, \text{ATP}_{\text{DNA}}, \text{ATP}_{\text{Lipid}}, \text{ATP}_{\text{Peptide}}, \text{ATP}_{\text{RNA}} \)  \( \) stoichiometric ATP demand for the synthesis of carbohydrates, DNA, lipids, proteins (including cellular proteins and product), and RNA, respectively, mmole ATP/cell

\( \text{ATP}_{\text{thr}}, \text{ATP}_{\text{val}} \)  \( \) stoichiometric ATP production from threonine and valine, respectively, mmole ATP/cell

\( \text{ATP}^d, \text{ATP}^f, \text{ATP}^n \)  \( \) stoichiometric ATP production from direct free energy release, phosphorylation of FADH$_2$, and phosphorylation of NADH, respectively, mmole ATP/cell

\( \text{ATP}^{\text{t}}, \text{ATP}^{\text{t}}, \text{ATP}^{\text{t}} \)  \( \) stoichiometric ATP demand for the transport of glucose and amino acids needed for cell mass and product synthesis (transport of nutrient for energy formation is not included), and the total stoichiometric ATP demand for transport, respectively, mmole ATP/cell

\( \text{ATP}^{\text{t}}, \text{ATP}^{\text{t}} \)  \( \) total stoichiometric ATP production and requirement for the synthesis for cell mass and product, respectively, mmole ATP/cell

\( C_{\text{amm}} \)  \( \) ammonia concentration in the supplemental medium, mM
C_{amm, n; C_{amm, n-1}} ammonia concentration in the nth and (n-1)th samples respectively, mM

C_i concentration of nutrient i in the supplemental medium, mM

C_k concentration of nutrient k in the supplemental medium, mM

C_k\textsuperscript{a} accumulative concentration of nutrient k added, mM

C_{k, n; C_{k, n-1}} concentration of glucose or glutamine in the nth and (n-1)th samples, respectively, mM

C_{lac, n; C_{lac, n-1}} lactate concentration in the nth and (n-1)th samples, respectively, mM

C_{p, n; C_{p, n-1}} product concentration in the nth and (n-1)th samples, respectively, mg/L

C_t total nutrient concentration in supplemental medium, mM

D_A, D_G, D_C, D_T DNA base compositions of adenine, guanine, cytosine, and thymine, respectively, molar percentage

F_i amount of reactants (i=1, 26) utilized per cell (negative values signify the amount of product produced from the reverse reaction), mmole reactant/cell

F_{5,a} amount of glutamate hydrolyzed through the reaction catalyzed by glutamate dehydrogenase, mmole glu/cell

F_{5,a}, F_{5,b} amount of glutamine amino group hydrolyzed and incorporated into NAAs, respectively, mmole/cell

F_{6,a}, F_{6,b} amount of glutamine amide group hydrolyzed and incorporated into nucleotides, respectively, mmole/cell

K_m Monod kinetic constant, mM

M_{a,i} molecule weight of peptide i

m_{ATP} maintenance ATP demand, mmole ATP/cell-h

M_{b}, M_{R} average molecular weight of the nucleotides in DNA and RNA, respectively

M_{CH_2}, M_{CH_2O}, M_{C4H4ON_3}, M_{C4H3O2N_2}, M_{C5H4N_5}, M_{C5H4O_2N_2}, M_{C5H7O}, M_{PO_4} molecular weight of units CH_2, CH_2O, C_4H_4O_3N_3, C_4H_3O_2N_2, C_5H_4N_5, C_5H_4O_5N_3, C_5H_5O_2N_2, C_5H_7O, PO_4, respectively
\( M^P_p, M^{PP}_p \) average molecule weights of peptides in cellular proteins and product, respectively

\( N_A, N_C, N_G, N_T, N_U \) total moles of adenine, cytosine, guanine, thymine, and uracil per cell, respectively, mmole/cell

\( N_{\text{DNA}}, N_{\text{RNA}} \) total moles of nucleotides in DNA and RNA per cell, respectively, mmole/cell

\( R_A, R_G, R_C, R_U \) RNA base compositions of adenine, guanine, cytosine, and uracil, respectively, molar percentage

\( RQ \) respiratory quotient, mmole CO\(_2\)/mmole O\(_2\)

\( \text{NADPH}_p, \text{NADPH}_r \) total production and demand of reducing power, respectively, mmole/cell

\( N_t \) total cell number in the reactor at time \( t \), number of cells

\( N_t^n \) total cell number calculated at the time when the \( n \)th sample was taken, number of cells

\( N_{t,0} \) total cell number in the reactor at the beginning of culture, number of cells

\( N_{t,n}; N_{t,n-1} \) total cells in the reactor when the \( n \)th and (\( n-1 \))th samples were taken, respectively, number of cells

\( N_v \) viable cell number in the reactor at culture time \( t \), number of cells

\( N_{v,n}; N_{v,n-1} \) viable cells in the reactor when the \( n \)th and (\( n-1 \))th samples were taken, respectively, number of cells

\( O^P_i, O^{PP}_i \) molar percentage of amino acid \( i \) in cellular proteins and product, respectively, percentage

\( \text{OUR} \) volumetric oxygen uptake rate, mmole O\(_2\)/L-h

\( P \) product concentration at time \( t \), mg/L

\( P_{\text{amm,n}} \) ammonia produced between the \( n \)th and (\( n-1 \))th samples, mmole

\( P_{l,n}, P_{l,n-1} \) amount of lactate or ammonia produced the \( n \)th and (\( n-1 \))th samples, respectively, mmole
$P_{\text{lac}, n}$ - lactate produced between the $n$th and $(n-1)$th samples, mmole

$P_{p, n}$ - total product generated between the $n$th and $(n-1)$th samples, mg

$q_{\text{ATP}}$ - specific ATP production rate, mmole/cell-h

$q_{\overline{i}}$ - average specific production rate of ammonia, lactate, or antibody respectively, mmole/cell-h (for ammonia and lactate), and mg/cell-h (for antibody)

$q_{p}$ - specific product synthesis rate, mg/cell-h

$S_{a, i}$ - stoichiometric synthesis of non-essential amino acid $i$, mmole/cell

$t$ - culture time, h

$t_n, t_{n-1}$ - culture time when the $n$th and $(n-1)$th samples were taken respectively, h

$U_{a, i}$ - stoichiometric uptake (negative signifies net production) of non-essential amino acid $i$, mmole/cell

$U_{a, i}^{\text{max}}$ - maximum stoichiometric uptake of non-essential amino acid $i$, mmole/cell

$U_{\text{ala}}, U_{\text{asn}}, U_{\text{asp}}, U_{\text{glu}}$ - stoichiometric consumption rate of alanine, asparagine, aspartate, and glutamine, respectively, mmole/cell

$U_{\text{glu}}, U_{\text{ile}}, U_{\text{leu}}, U_{\text{lys}}$ - stoichiometric consumption rate of glutamate, isoleucine, leucine, and lysine, respectively, mmole/cell

$U_{\text{pro}}, U_{\text{thr}}, U_{\text{val}}$ - stoichiometric consumption rate of proline, threonine, and valine, respectively, mmole/cell

$U_{\text{ala}}^{\text{max}}, U_{\text{asn}}^{\text{max}}, U_{\text{asp}}^{\text{max}}, U_{\text{glu}}^{\text{max}}, U_{\text{gly}}^{\text{max}}, U_{\text{pro}}^{\text{max}}, U_{\text{ser}}^{\text{max}}$ - maximum stoichiometric uptake of alanine, asparagine, aspartate, glutamate, glycine, proline, and serine, respectively, mmole/cell

$V_0$ - initial culture volume, L

$V_1$ - volume of supplemental medium fed to cells, L

$V_n, V_{n-1}$ - total volume in the reactor after the $n$th feeding and $(n-1)$th sample were taken, respectively, L
$V_R$  
volume of supplemental medium required for the production of $\Delta N_i$ cells, L

$V_{s,n}$, $V_{s,j}$, $V_{s,n-1}$  
volume of the nth, jth, and (n-1)th samples, respectively, L

$W$  
dry cell weight, mg/cell

$X_d$  
dead cell density at time t, cells/L

$X_i$, $Y_i$  
molar fractions (labeled in Figure 7.5) of the substrates attributed from glutamine and glucose ($i=1, 8$), respectively

$X_t$  
total cell density at time t, cells/L

$X_v$  
viable cell density at time t, cells/L

$X_{v,n}$, $X_{v,n-1}$  
viable cell density in the nth and (n-1)th samples, respectively, cells/L

$X_{t,0}$  
initial total cell density, cells/L

$X_{t,j}$, $X_{t,n}$  
total cell density in the jth and nth samples, respectively, cells/L

$Y_{\text{max}}^{\text{ATP}}$  
maximum cellular yield on ATP, cells/mmol ATP

$Y_{V\text{cell}}$  
ratio of the total ammonia or lactate production to the total production of cells, mmoles/cell

$Y_{\text{amn/gl}}$  
ratio of ammonia production to glucose consumption, mmol/mmol

$Y_{\text{ATP/gl}}$  
ATP yield on glucose, mmoles ATP/mole glucose

$Y_{\text{lac/gl}}^{\text{min}}$  
minimum molar ratio of lactate production to glucose consumption, mmol/mmol

$Y_{\text{lac/gl}}$  
molar ratio of lactate production to glucose consumption, mmol/mmol

$Y_{V,j}$  
cellular yield on vitamin j, cells/mmol vitamin

$Z_{C}, Z_{D}, Z_{L}, Z_{P}, Z_{R}$  
dry weight percentage of cellular carbohydrates, DNA, lipids, proteins, and RNA, respectively, in cell mass, percentage

$\Delta G_{\text{lc}}$  
total consumption of glucose, mmoles
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$\Delta \text{Lac}$</td>
<td>total production of lactate, mmole</td>
</tr>
<tr>
<td>$\Delta N_i$</td>
<td>total number of cells produced, number of cells</td>
</tr>
<tr>
<td>$\Delta N_{l,m}; \Delta N_{m,n}$</td>
<td>total cells produced since the initiation when the $n$th and $m$th samples were taken, respectively, number of cells</td>
</tr>
<tr>
<td>$\Delta V_{F,n}$</td>
<td>volume fed to the reactor between the $(n-1)$th and $n$th samples, L</td>
</tr>
<tr>
<td>$\Delta t$</td>
<td>time interval between two adjacent feeds, h</td>
</tr>
<tr>
<td>(\alpha)</td>
<td>specific cell death rate, 1/h</td>
</tr>
<tr>
<td>(\beta)</td>
<td>total stoichiometric coefficient of all nutrients in the supplemental medium, mmole/cell</td>
</tr>
<tr>
<td>$\delta_{\text{glc},n}; \delta_{\text{glu},n}$</td>
<td>Amount of glucose or glutamine, respectively, consumed between the $n$ and the $(n-1)$th sample, mmole</td>
</tr>
<tr>
<td>$\delta_{j,i}$</td>
<td>contribution of source $i$ to the production of product $j$, mmole product $j$/cell</td>
</tr>
<tr>
<td>$\delta_k$</td>
<td>total consumption of nutrient $k$ over the concerned time period, mmole</td>
</tr>
<tr>
<td>$\delta_{k,n}$</td>
<td>amount of glucose or glutamine consumed between the $n$th and the $(n-1)$th sample, mmole</td>
</tr>
<tr>
<td>$\eta_{F}, \eta_{N}$</td>
<td>number of ATP molecules produced from the complete oxidation of NADH and FADH$_2$, respectively</td>
</tr>
<tr>
<td>$\eta_i$</td>
<td>amount of source $i$, mmole/cell</td>
</tr>
<tr>
<td>$\phi_{j}$</td>
<td>total product of product $j$ from all sources, mmole/cell</td>
</tr>
<tr>
<td>$\bar{\mu}$</td>
<td>average cell specific growth rate over a concerned period of time, 1/h</td>
</tr>
<tr>
<td>$\mu$</td>
<td>specific cell growth rate, 1/h</td>
</tr>
<tr>
<td>$\theta_{\text{co}_2}$</td>
<td>stoichiometric carbon dioxide production, mmole/cell</td>
</tr>
<tr>
<td>$\theta_i$</td>
<td>stoichiometric coefficient of nutrient $i$ in the supplemental medium, mmole/cell</td>
</tr>
<tr>
<td>$\theta_{a,i}$</td>
<td>stoichiometric coefficient of amino acid $i$, mmole/cell</td>
</tr>
</tbody>
</table>
\( \theta_{a, i}^{\text{CP}} \) amino acid composition of cellular proteins, mmole/mg proteins

\( \theta_{a, i}^{\text{nuc}} \) stoichiometric demand (negative values signify production) of amino acid i for nucleotide synthesis, mmole/cell

\( \theta_{a, i}^{\text{P}} \) stoichiometric demand of amino acid i for protein (including product and cellular proteins) synthesis, mmole/cell

\( \theta_{a, i}^{\text{PP}} \) amino acid composition of product, mmole/mg product

\( \theta_{\text{ala}}, \theta_{\text{asn}}, \theta_{\text{asp}}, \theta_{\text{glu}}, \theta_{\text{glc}}, \theta_{\text{gly}}, \theta_{\text{pro}}, \theta_{\text{ser}} \)
stoichiometric coefficient of alanine, asparagine, aspartate, glucose, glutamate, glutamine, glycine, proline, and serine, respectively, including energy metabolism and biosynthesis from glutamine, mmole/cell

\( \theta_{\text{asp}}, \theta_{\text{glu}}, \theta_{\text{gly}}, \theta_{\text{ser}} \)
stoichiometric demand (negative values signify production) of aspartate, glutamine, glutamate, glycine, and serine, respectively, for nucleotide synthesis, mmole/cell

\( \theta_{\text{ala}}, \theta_{\text{asn}}, \theta_{\text{asp}}, \theta_{\text{glu}}, \theta_{\text{glc}}, \theta_{\text{gly}}, \theta_{\text{ser}} \)
stoichiometric demand of alanine, asparagine, aspartate, and glutamine for protein (including product) synthesis, respectively, mmole/cell

\( \theta_{\text{glu}}, \theta_{\text{ile}}, \theta_{\text{leu}}, \theta_{\text{lys}} \)
stoichiometric demand of glutamate, isoleucine, leucine, and lysine for protein (including product) synthesis, respectively, mmole/cell

\( \theta_{\text{pro}}, \theta_{\text{thr}}, \theta_{\text{val}} \)
stoichiometric demand of proline, threonine, and valine for protein (including product) synthesis, respectively, mmole/cell

\( \theta_{\text{ATP}} \) stoichiometric coefficient of ATP production in the overall stoichiometric equation, mmole ATP/cell

\( \theta_{\text{ATP}}^{\text{ATP}} \) stoichiometric demand of glucose for ATP production, mmole glucose/cell

\( \theta_{\text{o}_2} \) stoichiometric oxygen consumption, mmole/cell
\( \theta_p \)  
stoichiometric coefficient of product in the overall stoichiometric equation, mg/cell

\( \theta_{v,j} \)  
stoichiometric coefficient of the jth vitamin, mmole/cell

\( \tau \)  
integration of viable cells over culture time, cells-h

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ace</td>
<td>acetyl-CoA</td>
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<tr>
<td>amm</td>
<td>ammonia</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>cp</td>
<td>cellular proteins</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
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<tr>
<td>DMSO</td>
<td>dimethy sulfoxide</td>
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<tr>
<td>DO</td>
<td>dissolved oxygen</td>
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<tr>
<td>ELISA</td>
<td>enzyme-like immunosorption assay</td>
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<tr>
<td>F</td>
<td>feeding</td>
</tr>
<tr>
<td>FB</td>
<td>fed-batch culture experiment</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>glc</td>
<td>glucose</td>
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<tr>
<td>gln</td>
<td>glutamine</td>
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<td>glu</td>
<td>glutamate</td>
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<tr>
<td>G3P</td>
<td>glycerol-3-phosphate</td>
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<tr>
<td>GMP</td>
<td>good manufacturing practice</td>
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<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<tr>
<td>IMDM</td>
<td>Iscove's modified Dulbecco's medium</td>
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<tr>
<td>Init</td>
<td>initial medium</td>
</tr>
<tr>
<td>lac</td>
<td>lactate</td>
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<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>NAA</td>
<td>non-essential amino acid</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>nuc</td>
<td>nucleotides</td>
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<td>OPA</td>
<td>o-phenaldehyde</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>p</td>
<td>product</td>
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<td>pp</td>
<td>product protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>suc</td>
<td>succinyl-CoA</td>
</tr>
<tr>
<td>sup</td>
<td>supplemental medium</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
</tbody>
</table>
References


Appendixes

A: Program Code for Medium Design

```c
$debug

Function: calculate the coefficients for each nutrient
zp, zc, zl, zd, zr: dry cell weight percentage of protein,
carbohydrates, lipids, DNA, RNA (weight percentage)
W: dry cell weight (mg/cell)
Svit: coefficients for vitamins (micro mole/10^9 cells)
map: molecular weight of amino acid peptides
O%: molar percentage of amino acids in cellular proteins
u: specific growth rate (1/hr)
qP: specific product synthesis rate (mg/cell/hr)
Yatp, Map: qatp=u/Yatp + Map (cell/mmol, mmole/cell/hr)
Uala, Uasn, Uasp, Uglu, Ugly, Upro, User: specific uptake of
non-essential amino acids (mmole/cell)
Spa: stoichiometric coefficient of amino acid in cellular
protein synthesis reaction (mmole/mg protein)
Sa: stoichiometric coefficient of amino acid (mmole/cell)
Stf: content of Fe in cells (also stoichiometric coefficient)
(mmole/cell)
SP: stoichiometric coefficient of PO3 (mmole/cell)
Xa: molar fraction of amino acid in supplemental medium
Ca: concentration of amino acid in supplemental medium (mM)
Xvit: molar fraction of vitamin in supplemental medium
Cvit: concentration of vitamin in supplemental medium (mM)
MWaa: Molecular Weight of Amino Acids
MWvit: Molecular Weight of Vitamins
aaminae, vname: amino acid name and vitamin name
PO: P/O ratio

implicit real (m-n)
real Svit(10), map(20), o(20), Spa(20), Sa(20), Xa(20), Ca(20)
real Xvit(10), Cvit(10), MWvit(10), MWaa(20), Yaa(20), Yvit(10)
character aaminae(20)*3, vname(10)*20

open(10,file=' ',status='old')
open(20,file='coef.prm',status='new')

input data
read(10,*)Ct
read(10,*)Yiac, PO
read(10,*)Uala, Uasn, Uasp, Uglu, Ugly, Upro, User
read(10,*)u, qP, Yatp, Map, w0
read(10,*)zp, zc, zl, zd, zr, w

Adjust the energy requirement based on cell mass
Yatp=Yatp*w0*w
Map=Map*w0*w

Unify the fractions
zot=zp+zct+zlt+zdt+zrt
zp=zp/zot
zc=zc/zot
zl=zl/zot
zd=zd/zot
zr=zr/zot

read(10,*)Svit
do 1=1, 10
Svit(l)=Svit(l)*1.0e-12
1 continue

read(10,*)map
read(10,*)O
read(10,*)Da, Dg, Dc, Dt
```

256
read(10.*)Ra,Rg,Rc,Ru
read(10.*)aaame
read(10.*)vitname

read(10.*)CCa,CK,CMg,CNa
read(10.*)SFe

read(10.*)MWaa
read(10.*)MWvit
read(10.*)MWCa,MWK,MWMg,MWP,MWFe

calculate the molecular weight of each unit

mpo4=31.0+4.0*16.0
Mc5h7o=5.0*12.0+7.0+16.0
Mc5h7o2=5.0*12.0+7.0+2.0*16.0
ma=5.0*12.0+4.0+5.0*14.0
mg=5.0*12.0+4.0+18.0+5.0*14.0
Mc=4.0*12.0+4.0+16.0+3.0*14.0
Mt=5.0*12.0+5.0+2.0*16.0+2.0*14.0
Mu=4*12.0+3.0+2.0*16.0+2*14.0
mch2=12.0+2.0
mch2o=12.0+2.0+16.0
Md=Mpo4+Mc5h7o+Da*Ma+Dg*Mg+Dc*Mc+Dl*Mt
Mr=Mpo4+Mc5h7o2+Ra*Ma+Rg*Mg+Rc*Mc+Ru*Mu

calculate the amount (mmole) of DNA, RNA, and each bases per cell

Ndna=Zd*W/Md
Nrna=Zr*W/Mr
Na=Ndna*Da+Nrna*Ra
Ng=Ndna*Dg+Nrna*Rg
Nc=Ndna*Dc+Nrna*Rc
Nt=Ndna*Dl
Nu=Nrna*Du

calculate the stoichiometric coefficient of product

Sp=qp/u

calculate the average molecular weight of amino acid peptides

Mp=0.0
do 10 i=1,20
Mp=Mp+Map(i)*O(i)/100.0
continue

10

calculate the stoichiometric coefficient of each amino acid

in the synthesis of cellular proteins and product

do 20 i=1,20
Spa(i)=O(i)/100.0/Mp
Sa(i)=(Zp*W+Sp)*Spa(i)
continue

20

calculate the consumption of amino acids in the synthesis of DNA & RNA

Sa(6): gin; Sa(7): glu; Sa(4): asp; Sa(8): gly

Sa(6)=Sa(6)+3.0*Ng+2.0*(Na+Nc+Ni+Nu)
Sa(7)=Sa(7)-(3.0*Ng+2.0*(Na+Nc+Ni+Nu))
Sa(4)=Sa(4)+Ng+Na+Nu+Ni
Sa(8)=Sa(8)+Ng+2.0*Na

calculate the amount (mmole/cell) of non-essential amino acids synthesized from glutamine is calculated by subtracting the specific uptake from medium from the stoichiometric coefficient.

F4=Sa(1)-Uala
F9=Sa(3)-Uasn
F8=Sa(4)-Uasp+F9
F12=Sa(8)-Ugly

cF11=Sa(16)-User+F12
F5=F4+F8+F11

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F7=Sa(15)-Upro
F6=Sa(7)-Glu+F5+F7
write(",(5x, *F4-9,F11,F12"",/8(e16.4,2x))")
&
I4, I5, I6, I7, I8, I9, I11, I12

The stoichiometric coefficients of non-essential amino acids are then replaced by their real specific uptake. The stoichiometric coefficient of glutamine is then corrected by adding the amount consumed in the synthesis of non-essential amino acids.

Sa(1)=Uala
Sa(3)=Uasn
Sa(4)=Uasp
Sa(7)=Uglu
Sa(8)=Ugly
Sa(15)=Upro
Sa(16)=User
Sa(6)=Sa(6)+F6
do 25 i=1,20
if (Sa(i).le.0.0) Sa(i)=0.0
continue

The energy metabolism is considered in the following way: both glucose and glutamine contribute to energy production. When glutamine is consumed for non-essential amino acid synthesis, the carbon chain is assumed to enter TCA cycle for energy production. Lactate production from glucose and glutamine is also considered in the model.

Satp=1.0*Yatp+Matp/u
Scmglc=(Z/Mch2+Zc/Mch2o+5.0*Zd/Md+5.0*Zr/Mr)*W/6.0
&
Senglc=(Satp-(3.0+5.0*PO)*YSmglc+FI)*F1+Flac-(3.0+2.0*PO)*F5
&
-(3.0+4.0*PO)*F11)/(6.0+10.0*PO-(3.0+5.0*PO)*Ylac)
Senglc=(Satp+(3.0+4.0*PO)*YSmglc+FI1)*F11-Ylac-(3.0+2.0*PO)*F5
&
(8.0+8.0*PO-(3.0+4.0*PO)*Ylac)
Sglc=Scmglc+Senglc+FI11
write(",(5x,"Scmglc","i,1x, e16.4)"")Scmglc

beta is the total stoichiometric coefficient

beta=Scglc
do 30 i=1,20
beta=beta+Sa(i)
continue
do 40 i=1,10
beta=beta+Svl(i)
continue

molar fraction of each nutrient is calculated by dividing beta from their stoichiometric coefficient.

Xglc=Scglc/beta
Cglc=Xglc*Cl
Do 50 i=1,20
Xa(i)=Sa(i)/beta
Ca(i)=Xa(i)*Cl
continue
do 60 i=1,10
Xvl(i)=Svl(i)/beta
Cvl(i)=Xvl(i)*Cl
continue

calculate the requirement for salts

SP=Nnda*Nna
XPO4=SP/beta*2.0
XFe=SFb/beta
CP=XPO4*Cl
CFe=XFe*Cl

Yglc=Cglc*180.0/1000.0
Do 65 i=1,20
Yaa(i)=Ca(i)*MWaa(i)/1000.0

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continue
    do 66 i=1,10
      Yv(i)=Cv(i)*MWv(i)/1000.0
    continue
    YCa=CCa*MWCa/1000.0
    YK=CK*MWK/1000.0
    YMg=CMg*MWMg/1000.0
    YP=CP*MWP/1000.0
    YFe=CFe*MWFe/1000.0
    YNa=CNa*58.44/1000.0

    Write Report
    write(20,'('')')
    write(20,'('')')
    write(20,'(2x,"Cl= ",j6.1," mM", 5x,"YlAc/glC= ",j4.2,
    & 5x,"P/O= ",j3.1))Cl,YlAc,PO
    write(20,'(2x,"=",j6.4," 1/h",5x,"qP= ",e14.6,
    & " mg/cell-h")')u,qP
    write(20,'('')')
    write(20,'('')')

    write(20,'(2x,a13,5x,a16,6x,a5x,a3x,a)')'Nutrient Name',
    & 'mmole/10^9 cells', '%', 'mM', 'g/L'
    write(20,'(5x,a3,12x,f12.4,8x,0pf6.4,4x,f8,2,3x,F9.5)')
    & 'Glc','Sglc*1.0e9,Xglc,Cglc,Yglc
    write(20,'('')')
    do 70 i=1,20
      write(20,'(5x,a3,12x,0pf12.4,8x,0pf6.4,4x,f8,2,3x,F9.5)')
      & astrlen(i),Sv(i)*1.0e9,Xv(i),Cv(i),Yv(i)
    70 continue
    write(20,'('')')
    do 80 i=1,10
      write(20,'(5x,a15,112.5,7x,0pf7,5,4x,f8,3,3x,F9.5)')
      & astrlen(i),Sv(i)*1.0e9,Xv(i),Cv(i),Yv(i)
    80 continue
    write(20,'('')')
    write(20,'(5x,a4,11x,112.4)')'Beta',beta*1.0e9
    write(20,'('')')
    write(20,'(5x,"CaCl2",40x,f8,3,3x,F9.5)')CCa,YCa
    write(20,'(5x,"KCl",42x,f8,3,3x,F9.5)')OK,YK
    write(20,'(5x,"MgSO4",40x,f8,3,3x,F9.5)')CMg,YMg
    write(20,'(5x,"NaH2PO4",38x,f8,3,3x,F9.5)')CP,YP
    write(20,'(5x,"FeSO4",40x,f8,3,3x,F9.5)')CFe,YFe
    write(20,'(5x,"NaCl",41x,f8,3,3x,F9.5)')CNa,YNa
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    write(20,'('')')
    end
$debug
cimplicit real(l-n)
real lie
real map(20),o(20),Spa(20),Sa(20),A(20),UA(20)
real MWaa(20),PA(20),An(20),Asyn(7)
integer k(7)
character AA(20)*10,Title*60
open(10,file='",status='old'
open(30,file='",status='new'
open(40, file='",status='new'
read(10,"*"Title
read(10,"*"X,Y
read(10,"*"dNI,Glc,Lac,NH3
read(10,"*"A
read(10,"*"u,qp,Yatp,Matp,w0
read(10,"*"x2p,z2c,z2i,z2d,z2r,w
read(10,"*"map
read(10,"*"O
read(10,"*"Da,Dg,Dr,Dt
read(10,"*"Ra,Rg,Rc,Ru
read(10,"*"an
read(10,"*"AA
read(10,"*"k
Adjust the energy requirement based on cell mass
Yatp=Yatp/w0^w
Matp=Matp/w0^w
write(30,').')
write(30,'(1x,a)')Title
write(30,'(.')
write(30,'("P/O="4.13x,"Dry Cell Weight="e10.4)')X,W
write(30,'("dNI="e10.4,7x,"Growth Rate="f6.4)')dNI,u
write(30,'("Glc="e10.4,7x,"Lac="e10.4,5x,
& "NH3="e10.4)')Glc,Lac,NH3
write(30,'(.')
calculate the molecular weight of each unit
cmp4=31.0+4.0*16.0
Mcn=5.0*12+7.0+16.0
Mce=5.0*12+7.0+16.0
ma=5.0*12+4.0+5.0*14.0
mc=4.0*12+4.0+6.0+5.0*14.0
mt=5.0*12+5.0+2.0*16.0+2.0*14.0
Mu=4*12+3.0+2.0*16.0+2*14.0
mch2=12.0+2.0
Md=Mpo4+Mc5h7o+Da*Ma+Dg*Mg+Dc*Mc+Di*Mt
Mr=Mpo4+Mc5h7o+Ra*Ma+Rg*Mg+Rc*Mc+Ru*Mu
Mch=100*5.0*12+10.0+16.0*5.0
calculate the amount (mmole) of DNA, RNA, and each bases per cell
Ndn=Zd*W/Md
Nrra=Zr*W/Mr
Na=Ndn*Da+Nrra*Ra
Ng=Ndn*Dg+Nrra*Rg
Nc=Ndn*Dc+Nrra*Rc
Nt=Ndn*Di
Nu=Nrra*Du
calculate the stoichiometric coefficient of product
Sp=qp/u
calculate the average molecular weight of amino acid peptides
Mp=0.0
do 10 i=1,20
calculate the stoichiometric coefficient of each amino acid
in the synthesis of cellular proteins and product
Calculate the average molecule weight of peptides

\[\text{do } 20 \text{ } i=1,20\]
\[\text{Spa}(i)=O(i)/100.0/Mp\]
\[\text{Sa}(i)=(Zp)^*W+Sp)^*\text{Spa}(i)\]

add the consumption of amino acids in the synthesis of DNA & RNA

\[\text{Sa}(6)=\text{Sa}(6)+3.0^*\text{Ng}+2.0^*\text{Na}+\text{Nc}+\text{Nu}\]
\[\text{Sa}(4)=\text{Sa}(4)+\text{Ng}+2.0^*\text{Na}+\text{Nc}+\text{Nu}\]
\[\text{Sa}(8)=\text{Sa}(8)-\text{Na}-\text{Ng}\]
\[\text{Sa}(16)=\text{Sa}(16)+\text{Nt}+2.0^*\text{Na}+2.0^*\text{Ng}\]

\[\text{do } 30 \text{ } i=1,20\]
\[\text{UA}(i)=A(i)/dNt\]
\[\text{if}(1.\text{eq}5.0\text{or}.1.\text{eq}9)\text{UA}(i)=\text{Sa}(i)\]

\[\text{aa}(20)(4:4)=+^*\]
\[\text{aa}(20)(5:7)=\text{aa}(18)\]
\[\text{aa}(17)(4:4)=+^*\]
\[\text{aa}(17)(5:7)=\text{aa}(2)\]

\[\text{F2}=\text{Lac/dNt}\]
\[\text{Sglc} = \text{Glc/dNt}\]
\[\text{F14} = \text{UA}(12)-\text{Sa}(12)\]
\[\text{F15} = \text{UA}(10)-\text{Sa}(10)\]
\[\text{F16} = \text{UA}(11)-\text{Sa}(11)\]
\[\text{F4} = \text{UA}(1)+\text{Sa}(1)\]
\[\text{F7} = \text{UA}(15)+\text{Sa}(15)\]
\[\text{F9} = \text{UA}(3)+\text{Sa}(3)\]
\[\text{F8} = \text{UA}(4)+\text{Sa}(4)+\text{F9}\]

\[\text{F21} = \text{UA}(17)-\text{Sa}(17)+\text{UA}(2)-\text{Sa}(2)\]
\[\text{F22} = \text{UA}(20)-\text{Sa}(20)+\text{UA}(18)-\text{Sa}(18)\]

\[\text{if}(\text{F14}.\text{It}0.0)\text{F14}=0.0\]
\[\text{if}(\text{F15}.\text{lt}0.0)\text{F15}=0.0\]
\[\text{if}(\text{F16}.\text{lt}0.0)\text{F16}=0.0\]
\[\text{if}(\text{F21}.\text{lt}0.0)\text{F21}=0.0\]
\[\text{if}(\text{F22}.\text{lt}0.0)\text{F22}=0.0\]
\[\text{F17} = \text{Zd}^*W/Md+\text{Zr}^*W/Mr\]
\[\text{F18} = \text{Zc}^*W/Mc6h1005\]
\[\text{F19} = \text{Z}^*W/Mch2/4.0\]
\[\text{F12} = \text{UA}(8)+\text{Sa}(8)\]
\[\text{F11} = (-\text{UA}(16)+\text{Sa}(16)+\text{F12})/2.0\]
\[\text{F6} = \text{UA}(6)-\text{Sa}(6)+3.0^*\text{Ng}+2.0^*(\text{Na}+\text{Nc}+\text{Nu})\]
\[\text{F5} = \text{F6}+\text{UA}(7)-\text{F7}-\text{Sa}(7)\]
\[\text{F5a} = \text{F5}+\text{F4}-\text{F8}+2.0^*\text{F11}\]
\[\text{F20} = (\text{Z}^*W/Mch2/2.0+\text{Ndna}^*(2.0^*\text{Dl}+\text{Dc}+2.0^*\text{Da})\]

\[+2.0^*\text{NA}^*(1+\text{Rg}+\text{Ra})]/12.0\]
\[\text{F1} = \text{Glc/dNt-F11-F17-F18-F19-F20}\]
\[\text{F25} = 2.0^*\text{Na}+\text{Ng}\]
\[\text{F3} = 2.0^*\text{F1}-\text{F2}-\text{F4}-\text{F8}+2.0^*\text{F14}+\text{F5}+2.0^*\text{F15}+3.0^*\text{F16}+\text{F21}+\text{F22}+\text{F25}\]
\[\text{F23} = \text{F3}+\text{F5}\]
\[\text{F24} = \text{F23}+\text{F15}+\text{F21}+\text{F22}\]
\[\text{F26} = \text{F25}+\text{F24}\]
\[\text{F10} = \text{F26}-\text{F3}-\text{F8}\]
\[\text{F13} = 2.0^*\text{F1}+\text{F10}-\text{F2}-\text{F4}\]

\[\text{F} = \text{F10}+2.0^*\text{F1}\]
\[\text{F0} = \text{F26}+\text{F8}\]
\[\text{if}(\text{F8}.\text{GE}.0.0)\text{then}\]
\[\text{X1} = (\text{F5}/\text{F26}+\text{F1}^*\text{F10}/\text{F3}/\text{F3}/(\text{F2.0}^*\text{F3}/\text{F26}-\text{F10}^*\text{F13}/\text{F26}/\text{F})\]
\[\text{Y1} = (2.0^*\text{F1}^*\text{F13}/\text{F3}/\text{F})/(2.0^*\text{F3}/\text{F26}-\text{F10}^*\text{F13}/\text{F26}/\text{F})\]
\[\text{else}\]
X1=(F5/F0+F1*F10/F3/F0)/(2.0*F3/F0-F10*F13/F0/F0)
Y1=(2.0*F1*F13/F3/F0)/(2.0*F3/F0-F10*F13/F0/F0)
endif
X2=(X1*F3+F5)/F23
Y2=Y1*F3/F23
X3=X2*F23/F24
Y3=Y2*F23/F24
X4=X3*F24/F26
Y4=Y3*F24/F26
if(F8.ge.0.0) then
  X5=X4
  X6=X4
  Y5=Y4
  Y6=Y4
else
  X5=0.0
  Y5=0.0
  X6=X4*F26/F0
  Y6=Y4*F26/F0
endif
X7=(Y6*F10+2.0*F1)/F
Y7=(Y6*F10+2.0*F1)/F
X8=X7*F13/F3
Y8=Y7*F13/F3

F5a1=F5a
if(F5a1.le.0.0)F5a1=0.0
ATPglc=(2.0*F1+Y3*F24)+(Y7*F13+Y1*F3+Y2*F23+Y4*F26)*X
&+(2.0*F1-Y7*F2+Y3*F24)*Y
ATPgln=(X3*F24+(X2*F23+X4*F26+X7*F13+X1*F3+F5a1)*X
&+(X3*F24-X7*F2)*Y
ATPase=1.0+3.0*Y+X
ATPpsuc=2.0+5.0*X+2.0*Y
ATPile=1.5*(3.0*X+Y-1.0+ATPace+ATPpsuc)
ATPleu=16*(2.0*X+Y-2.0+3.0*ATPace)
ATPlys=1.4*(5.0*X+Y+2.0*ATPace)
ATPthr=21*(X-1.0+ATPpsuc)
ATPval=22*(4.0*X+Y-1.0+ATPpsuc)

Energy Balance

ATPd=2.0*F1+F24-F7-F15-2.0*F16-F17-F18
ATPn=(F3+15+4.0*F11+13+5.0*F14+3.0*F15+2.0*F16+F21+F22
&+F23+F26)*X
ATPi=(2.0*F1-F2+F14+F15+F24)*Y
Patp=ATPd+ATPn+ATPi

ATTP=(Zp*W+Sp)*4.0/Mp
ATPC=F18*2.0
ATPI=(4.0-X-Y)*F1R
ATP=Na*(Cu*+Rc*(7.0-X)+8.0*Ra+Rg*(9.0-X)+1.0)
ATPD=Ndna*(Dc'*(7.0-X)+Dl'*(8.0-X)+8.0*Da+Dg*(9.0-X)+1.0)
Ratsp=ATPp+ATPC+ATPi+ATPr+ATPd

Ninput+Glc+dNt+(Zp*W+Sp)/Mp+F6+F14+F15+F16+F21+F22
ATPout=F4
ATP=ATPlin+ATPout

Nitrogen Balance

Ninput=0.0
do 40 i=1,20
  if(UA(i).le.0.0) go to 40
  Ninput=Ninput+UA(i)*AN(i)
40 continue
Np=0.0
do 50 i=1,20
  Np=Np+(Zp*W+Sp)*Spa(i)*AN(i)
50 continue
Nn=5.0*Na+4.0*Ng+2.0*Nl+3.0*Nc+2.0*Nu
Naa=0.0
Do 52 i=1,20
if(UA(i).ge.0.0) go to 52
Naa=Naa-UA(i)*An(i)
continue
Noutput=Np+Nn+NH3/dNt+Naa
CO2=F3+F10+F13+2.0*F14+I17+2.0*F19+6.0*F20+F22+F23-Na-Ng
Satp=1.0/Yatp+Matp/u
glutamine & Non-Essential Amino Acids Balance
Nitrogen Balance
Ngln=2.0*UA(6)
Ngln=2.0*Spa(6)*Zp*W+Sp
Ngln=3.0*Ng+2.0*(Na+Nc+Ni)+Nu-Dt*Ndna
Ngln=0.0
Do 55 i=1,7
if(Sa(i).gt.UA(i))
 Nglna=Nglna+(Sa(i)-UA(i))*An(i)
continue
Amino group transferred from glutamine
TNH3=0.0
if(F4.ge.0.0)TNH3=TNH3+F4
if(F8.ge.0.0)TNH3=TNH3+F8
if(F11.ge.0.0)TNH3=TNH3+F11*2.0
Nglnh3=0.0
if(F9.gt.0.0) then
 Nglnh3=F6-F9-(3.0*Ng+2.0*(Na+Nc+Ni)+Nu)+Dt*Ndna
else
 Nglnh3=F6-(3.0*Ng+2.0*(Na+Nc+Ni)+Nu)+Dt*Ndna
endif
Both Gin and Ess. A.A. can contribute to Non-ess. A.A.
and Nh3 production.
GLUess=2.0*F14+F15+F16+F21+F22
if((GLUess+F5).ge.TNH3) then
 if(Sa(7).gt.UA(7)) then
 Glupool=F6+GLUess
 else
 Glupool=F6+GLUess+UA(7)-Sa(7)
endif
SYNess=TNH3*GLUess/Glupool
NH3ess=GLUess-SYNess
else
 NH3ess=0.0
SYNess=GLUess
endif
NH3TCA=F5+GLUess-TNH3
Ngln=Ngln-SYNess
Nglnnh3=Nglnnh3+NH3TCA-NH3ess
NH3tot=NH3ess -Nglnnh3
Nginout=Ngln+Nglnh+Nglna+Nglnnh3
Carbon Balance for Glutamine
Cginln=UA(6)
Cgin=0.0
Cginp=Spa(6)*Zp*W+Sp
if(Sa(7).LE.UA(7)) then
 CginTCA=F5-(UA(7)-Sa(7))
else
 CginTCA=F5
 Cgin=3glna+Sa(7)-UA(7)
endif
Cgin=3glna+Sa(15)-UA(15)
if(Sa(15).LE.UA(15)) Write(30,'(" Wrong in Proline")')
Cginout=Cginp+CglnTCA+Cgln
Glucose Balance
write(30,'(" Glutamine Balance: Nitrogen")')
write(30,'(" Total input="e,10.4")Nglnin
write(30,'(" Total output="e,10.4")Nglnout
write(30,'(" Protein =e,10.4,f14.2,"%")')
& Nglnp,Nglnp/Nglnout*100.0
write(30,'(" DNA & RNA =e,10.4,f14.2,"%")')
& Nglnm,Nglnm/Nglnout*100.0
write(30,'(" Non-ESS AA="e,10.4,f14.2,"%")')
& Nglnh3,Nglnh3/Nglnout*100.0
write(30,'( )
write(30,'(" Glutamine Balance: Carbon")')
write(30,'(" Total input="e,10.4")Cglnin
write(30,'(" Total output="e,10.4")Cglnout
write(30,'(" Protein =e,10.4,f14.2,"%")')
& Cglnp,Cglnp/Cglnout*100.0
write(30,'(" TCA Cycle="e,10.4,f14.2,"%")')
& CglnTCA,CglnTCA/Cglnout*100.0
write(30,'(" Non-Ess.AA="e,10.4,f14.2,"%")')
& Cglna,Cglna/Cglnout*100.0
write(30,'( )
write(30,'(" Glucose balance:"))
write(30,'(" Total Glucose input="e,10.4")Cgt
write(30,'(" Total Glucose output="e,10.4")Cgt
write(30,'(" Carbohydrates =e,10.4,F10.2,"%")')
& F18,F18/Cgt*100.0
write(30,'(" Liplds =e,10.4,F10.2,"%")')
& F19,F19/Cgt*100.0
write(30,'(" DNA & RNA =e,10.4,F10.2,"%")')
& F17,F17/Cgt*100.0
write(30,'(" Reducing Power =e,10.4,F10.2,"%")')
& F20,F20/Cgt*100.0
write(30,'(" Serine & Glycine =e,10.4,F10.2,"%")')
& F11,F11/Cgt*100.0
write(30,'(" Glycolytic Pathway="e,10.4,F20.2,"%")')
& F11,F11/Cgt*100.0
write(30,'(" Lactate =e,10.4,F14.2,"%")')
& Clac,Clac/Cgt*100.0
write(30,'(" Alanine =e,10.4,F14.2,"%")')
& F42,F42/Cgt*100.0
write(30,'(" TCA Cycle="e,10.4,F14.2,"%")')
& CTCA,CTCA/Cgt*100.0
write(30,'( )
write(30,'(1.x,"F1 - F5=key,5(e,12.4)")F1,F2,F3,F4,F5
write(30,'(1.x,"F6 -F10=key,5(e,12.4)")F6,F7,F8,F9,F10
write(30,'(1.x,"F11-F15=key,5(e,12.4)")F11,F12,F13,F14,F15
write(30,'(1.x,"F16-F20=key,5(e,12.4)")F16,F17,F18,F19,F20
write(30,"(1.x,"F21-F25=key,5(e,12.4)")F21,F22,F23,F24,F25
write(30,"(1.x,"F26,F5a=key,5(e,12.4)")F26,F5a
write(30,"( )")
write(30,"( X1-X8=key,2x,8(f5.3,3x))")X1,X2,X3,X4,X5,X6,X7,X8
write(30,"( Y1-Y8=key,2x,8(f5.3,3x)")Y1,Y2,Y3,Y4,Y5,Y6,Y7,Y8
WRITE(30,'( )
write(30,'(" Energy Balance")')
write(30,'(" Total Energy Produced="e,10.4")Patp
write(30,"(" Glic="e,10.4,f14.2,"%")")
& ATPgil,ATPgil/Patp*100.0
write(30,"(" Gin="e,10.4,f14.2,"%")")
& ATPgim,ATPgil/Patp*100.0
write(30,"(" Leu="e,10.4,f14.2,"%")")
& ATPleu,ATPileu/Patp*100.0
write(30,"(" Ile="e,10.4,f14.2,"%")")
& ATPile,ATPileu/Patp*100.0
write(30,"(" Lys="e,10.4,f14.2,"%")")
& ATPlys,ATPlys/Patp*100.0
write(30,"(" Val="e,10.4,f14.2,"%")")
& ATPval,ATPval/Patp*100.0
write(30,"(" Thr="e,10.4,f14.2,"%")")
& ATPthr,ATPthr/Patp*100.0
write(30,"(" Tyri="e,10.4,f14.2,"%")")
& ATPtyr,ATPtyr/Patp*100.0
write(30,"(" Total Energy Required="e,10.4")Satp
write(30,"(" Total Energy for Synthesis="e,10.4,"%")")
& f14.2,"%")) Ratps,Ratps/Satp*100.0
& write(30,(" Protein=",e10.4,f14.2,"%"))
& ATP1,ATP2/Ratp*100.0
& write(30,(" Carboy=",e10.4,f14.2,"%"))
& ATPc,ATPb/Ratp*100.0
& write(30,(" Lipids=",e10.4,f14.2,"%"))
& ATPi,ATPI/Ratps*100.0
& write(30,(" RNA=",e10.4,f14.2,"%"))
& ATPr,ATPr/Ratps*100.0
& write(30,(" DNA=",e10.4,f14.2,"%"))
& ATPd,ATPd/Ratps*100.0

c
& write(30,(" Total Energy for Transport=",e10.4,
& f14.2,"%")) ATPi,ATPl/ATp*100.0
& write(30,(" Transport in=",e10.4,f14.2,"%"))
& ATPin,ATPlin/ATpin*100.0
& write(30,(" Transport out=",e10.4,f14.2,"%"))
& ATPout,ATPout/ATp*100.0
& write(30,(" Total CO2 Production=",2e12.4)) CO2,CO2*dN
& write(30,(" ))

d 1000 l=1.15
& write(30,(" ))
1000 continue
write(40,("(1x, f8.4))F1*1e9
write(40,("(1x, f8.4))F2*1e9
write(40,("(1x, f8.4))F3*1e9
write(40,("(1x, f8.4))F4*1e9
write(40,("(1x, f8.4))F5*1e9
write(40,("(1x, f8.4))F6*1e9
write(40,("(1x, f8.4))F7*1e9
write(40,("(1x, f8.4))F8*1e9
write(40,("(1x, f8.4))F9*1e9
write(40,("(1x, f8.4))F10*1e9
write(40,("(1x, f8.4))F11*1e9
write(40,("(1x, f8.4))F12*1e9
write(40,("(1x, f8.4))F13*1e9
write(40,("(1x, f8.4))F14*1e9
write(40,("(1x, f8.4))F15*1e9
write(40,("(1x, f8.4))F16*1e9
write(40,("(1x, f8.4))F17*1e9
write(40,("(1x, f8.4))F18*1e9
write(40,("(1x, f8.4))F19*1e9
write(40,("(1x, f8.4))F20*1e9
write(40,("(1x, f8.4))F21*1e9
write(40,("(1x, f8.4))F22*1e9
write(40,("(1x, f8.4))F23*1e9
write(40,("(1x, f8.4))F24*1e9
write(40,("(1x, f8.4))F25*1e9
write(40,("(1x, f8.4))F26*1e9
end