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GLUTAMINE REPLENISHMENT AND AMMONIA REMOVAL IN HYBRIDOMA CELL CULTURES VIA IMMOBILIZED GLUTAMINE SYNTHETASE

by

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ABSTRACT

Hybridoma cells utilize glutamine as their primary nitrogen source and excrete ammonia as a metabolic waste product. This ammonia can quickly accumulate to toxic levels in hybridoma culture media, and can severely reduce monoclonal antibody production (Ozturk et al., 1991). The enzyme glutamine synthetase (E.C. 6.3.1.2), which catalyzes the reaction

$$NH_4^+ + \text{L-glutamate} + ATP \xrightarrow{Mg^{2+}} \text{L-glutamine} + ADP + P_i + H^+,$$

was evaluated as a means of reducing ammonia and replenishing glutamine in hybridoma culture medium.

The effect of each enzyme reactant on soluble glutamine synthetase activity was quantified, and enzymatic reaction equilibrium evaluated. Enzyme reaction rates in two culture media, both with and without serum, were compared. Glutamine synthetase was immobilized via three different methods, and their effects compared. Cell sensitivity to each enzymatic reactant was studied. Finally, immobilized glutamine synthetase was incorporated in a hybridoma cultivation, and its effect on culture characteristics evaluated.

Chapter 1 – Introduction and Literature Review

Monoclonal Antibodies

Monoclonal antibodies (MAb's) play a critical role in the immune response of higher vertebrates. Produced by Effector B lymphocytes, or plasma cells, these antibodies bind specifically to only one epitope, or antigenic determinant, on the surface of an antigen. Antigens thus bound are inactivated and precipitate from solution (Mathews and van Holde, 1996).

Monoclonal antibody molecules are composed of two heavy chains and two light chains which form a Y-shape. These heavy and light chains have molecular weights of approximately 53,000 and 23,000 each, respectively, and are held together by disulfide bonds. The base and lower portion of each branch of the "Y" is called the Fc, or complement-binding domain. The structure of this region is the same for all antibodies of a particular class. The upper portion of each branch of the "Y" is called the Fab, or antigen-binding domain. This region varies in primary, secondary and tertiary structure from MAb to MAb; these differences yield binding sites for different epitopes, and provide each MAb its specificity (Mathews and van Holde, 1996).

In addition to their *in vivo* immune system role, monoclonal antibodies are used in several industrial applications which rely on their binding specificity. They are used extensively in immunoassays, which measure the amount of some antigen of interest in a sample. In this process, the sample is typically added to a small chamber containing

immobilized MAb's, and to which the antigens to be quantified in the sample then bind (Wild and Davies, 1994). The resulting antibody-antigen complexes are then labeled. In radioimmunoassays, or RIA's, the complexes are labeled with a radioactive isotope, and the resulting signal read by a scintillation counter. In enzyme immunoassays (EIA's), also referred to as enzyme-linked immunosorbent assays (ELISA's), the complexes are labeled with an enzyme, which catalyzes a change in color, fluorescence, or chemiluminescence in solution that is quantifiable and scales with sample antigen concentration (Wild and Davies, 1994). Monoclonal antibodies are also used for purification, via affinity chromatography, and for some therapeutic applications.

Although monoclonal antibodies were first recognized in the 1950's, attempts at producing significant quantities via tissue culture of unmodified plasma cells were largely unsuccessful (Deshpande, 1996). In 1975, Kohler and Milstein provided a major advance in MAb production by developing the process to generate hybridoma cells. These cells are comprised of a MAb-producing spleen cell fused to a myeloma cell, which immortalized the spleen cell and allowed long-term antibody production (Wild and Davies, 1994).

Hybridomas are employed to continuously produce monoclonal antibodies by two primary methods: *in vivo* via mouse ascites, and *in vitro* hybridoma cell culture. In the first method, hybridomas that secrete the desired product are injected into the peritoneal cavities of mice or rats. These cavities serve as growth chambers, and, as the hybridomas proliferate, tumors form. Fluid from these tumors, called ascites fluid, is harvested; this fluid contains up to 5-15 mg/ml of antibody, and each mouse or rat can yield approximately 50-100 mg of antibody.

The mouse ascites method provides a concentrated antibody product, typically between 0.9 and 9 mg/ml of the desired antibody, which reduces the need for subsequent concentration prior to use (Harlow and Lane, 1988). However, this method does have some disadvantages. First, the desired antibody is often contaminated with other mouse antibodies in ascites fluid, necessitating expensive product purification. Second, mouse ascites is largely ineffective in producing human antibodies, which typically provide better results in human therapeutics (Campling et al., 1987). Third, the ethical implications of mouse ascites have also recently been called into question, with some opposed to the use of mice as "antibody factories". Fourth, this method cannot easily be scaled up for production of large quantities of MAb's.

In Vitro *Hybridoma Culture*

In vitro hybridoma culture does not provide as concentrated of a MAb product as mouse ascites, and therefore often requires concentration prior to industrial use. Concentrations generally reach only 0.05 mg/ml of the desired antibody (Harlow and Lane, 1988). However, *in vitro* culture does offer easier scale-up, and often produces a purer antibody product than mouse ascites, which can reduce purification costs. Also, human MAb-producing hybridomas are easier to culture *in vitro*, which makes this technique substantially more flexible than mouse ascites (Campling et al., 1987).

Hybridoma cells are typically cultured *in vitro* in liquid culture medium, either immobilized within a porous matrix, such as an agarose gel, or in suspension. This culture medium supplies the cells with nutrients, minerals, vitamins and proteins necessary to

support cell growth. The compositions of two commercially available media are provided in Appendix 1. Culture media are often supplemented with animal blood serum to provide additional compounds needed by the cells. These compounds include additional nutrients, growth factors, adhesion proteins such as albumin and transferrin, and non-specific protection factors against mechanical damage. Also gaining acceptance is the use of serum-free media, which generally consist of some combination of commercial media supplemented with known concentrations of certain blood serum compounds found to enhance cell growth and antibody production. Antibody produced via serum-free cultivation is usually purer than that from serum-supplemented cultivation due to its lack of extraneous serum antibodies and other proteins (Maurer, 1992).

The metabolism of hybridoma cells is fairly complex. Hybridomas utilize glucose and glutamine as their primary carbon, nitrogen and energy sources (Miller et al., 1988). Ozturk and Palsson (1992) observed that hybridoma cell growth ceased upon culture medium glutamine exhaustion, and glucose could not substitute for glutamine. Miller and coworkers (1988) also reported a decline in viable cell density upon glutamine depletion. In addition, they observed that the extracellular antibody concentration increased with increasing glucose and glutamine, which they attributed to an increase in viable cell density, not an increase in specific antibody production rate.

The metabolism of glucose and glutamine primarily produce lactate and ammonia, respectively, as waste products, both of which can accumulate to toxic levels in culture media. Of the two, lactate is less toxic to hybridoma cells. Studies involving different cell lines observed no cell growth-rate inhibition with an initial lactate concentration of 40 mM

(Miller et al., 1988), and 50% inhibition at an initial concentration of 55 mM (Ozturk et al., 1992).

Ammonia accumulation in culture media, from both cellular metabolism and spontaneous deamination of glutamine, produces toxic effects at much lower concentrations. Ozturk and coworkers (1992) observed a 50% reduction in specific cell growth rate from initial ammonia concentrations as low as 4 mM. Other groups studying different mouse hybridoma cell lines have observed similar adverse effects at 5 mM (Glacken, 1987), 3 mM (Reuveny et al., 1987), and 1.8 mM ammonia (Truskey et al., 1990). Ozturk and coworkers also reported a decrease in total antibody production with increasing ammonia due to a lowered cell density, but likely not due to a decrease in specific production rate.

Methods of Ammonia Removal From Hybridoma Cell Culture

Due to the well documented adverse effect of ammonia accumulation in *in vitro* hybridoma culture, several methods have been employed to either remove ammonia from media or otherwise prevent its accumulation. The most common method is probably media perfusion. In this method, culture medium within a bioreactor is continuously replaced with fresh medium to provide continuous nutrient supplementation and to slow ammonia accumulation (Nayve et al., 1991). Although simple, this method does present some disadvantages. First, perfusion requires the use of large volumes of culture medium, which can result in substantial cost. Also, spent medium is often filtered prior to removal to keep hybridoma cells and monoclonal antibodies within the bioreactor. These filters

can foul rapidly, which necessitates either frequent backflushing or filter replacement (Nayve et al., 1991).

Other methods of *in situ* ammonia removal/prevention have also been investigated. Capiaumont and coworkers (1995) evaluated three different approaches. The first approach, replacing glutamine with glutamate in culture media to prevent ammonia production, yielded reduced cell densities illustrating that glutamine was absolutely essential for hybridoma cell growth. The other two approaches, selective removal of ammonia with a cation-exchange resin (clinoptilolite) and passage of the medium through a microporous hollow fiber module, were both effective at maintaining ammonia at low concentrations, but no changes in specific antibody production were observed for either. Nayve and coworkers (1994) employed zeolite beads as a cation exchanger to remove ammonia from culture medium from which both cells and antibody had been filtered. This approach was temporarily successful and high cell densities (up to 2.5×10^7 cells/ml) were attained. However, both the ultrafiltration membrane and ceramic filter incorporated upstream of the zeolite column were quickly fouled, and frequent backflushing was necessary.

Paredes and coworkers (1999) attempted to prevent extracellular ammonia accumulation by transfecting hybridoma cells with the gene for glutamine synthetase, an enzyme involved in ammonia assimilation that is lacking in hybridomas. This method was effective in essentially eliminating ammonia accumulation within the culture medium. However, such an approach has the limitations that the cell metabolism is altered, and that any future cell lines would requires similar transfection.

Escherichia coli *Glutamine Synthetase*

The enzyme glutamine synthetase is found in a wide gamut of organisms, from bacteria to humans. Although its characteristics vary from species to species, it plays the same critical role in each: the assimilation of ammonia and the synthesis of the amino acid glutamine, a vital cell nutrient. Due to the importance of glutamine for cell metabolism and the potential toxicity of ammonia at relatively low concentrations, glutamine synthetase is intricately regulated *in vivo* via two primary mechanisms. Its unique regulation cascade has made glutamine synthetase the subject of much study.

The glutamine synthetase of *E. coli* is dodecameric in structure. It is comprised of twelve identical subunits arranged in two stacked, identical hexagonal rings. Each subunit has a formula weight of approximately 50,000, which yields a total molecular weight for the enzyme of approximately 600,000 (Maurizi and Ginsburg, 1985).

Each subunit contains an identical active site which is located at the border with an adjacent subunit on the same hexagonal ring. Each active site includes two divalent cation binding sites: one stronger binding site, often called n_1, and a weaker binding site, called n_2. X-ray crystallography suggests that n_1 is located at or near His 210 and n_2 at His 269 (Li et al., 1988). Binding of divalent cations, particularly Mg^{2+}, Mn^{2+}, or Co^{2+}, to the n_1 site affects a conformational change, easily discernible via spectroscopy, from the "relaxed" (catalytically inactive) form to the "taut" (active) form (Denton and Ginsburg, 1970). This conformational shift involves the transfer of 12-24 tryptophan and tyrosine residues from polar to non-polar environments (Segal and Stadtman, 1972). The n_2 site is

involved in phosphoryl transfer and the binding of nucleotides (Maurizi and Ginsburg, 1985).

Of the three divalent cations mentioned above, binding of Mg^{2+} at sites n_1 and n_2 provides the highest enzyme catalytic activity under most circumstances (Woolfolk et al., 1966). However, Mn^{2+} and Ca^{2+} bind much more tightly to these sites, probably due to the fact that Mg^{2+} is preferentially solvated due to its smaller ionic radius (Abell and Villafranca, 1991). For this reason, Mn^{2+} and Ca^{2+}, as well as Cd^{2+} and Zn^{2+}, have been observed to be potent inhibitors of Mg^{2+}-activated glutamine synthetase activity. In contrast, the addition of Co^{2+} has been observed to enhance enzyme activity under some conditions (Segal and Stadtman, 1972).

Glutamine synthetase catalyzes the reaction

$$\text{L-glutamate} + NH_4^+ + \text{ATP} \xrightarrow{M^{2+}} \text{L-glutamine} + \text{ADP} + P_i + H^+,$$

where M^{2+} is one of the divalent cations previously mentioned and P_i is inorganic phosphate. This reaction likely follows a preferred ordered pathway, in which, in the case of Mg^{2+}-activated enzyme, the Mg-ATP complex first binds to the n_2 site. Strong evidence suggests that during the reaction a γ-glutamyl phosphate intermediate is formed according to the equation

$$\text{Mg-ATP} + \text{Glu} \rightarrow \text{Mg-ADP-}\gamma\text{-Glu-P},$$

where Glu is glutamate, followed by the displacement of phosphate by ammonia according to the equation

$$Mg\text{-}ADP\text{-}\gamma\text{-}Glu\text{-}P + NH_3 \rightarrow MgADP + Gln + P_i,$$

where the release of the resulting Mg-ADP complex by the n_2 site is the rate limiting step (Midelfort and Rose, 1976, Weisbrod and Meister, 1973). The overall reaction exhibits strong specificity for the L-isomer of glutamate; replacement with D-glutamate yields only 5% activity (Woolfolk et al., 1966).

Different divalent cations as cofactors seem to effect enzyme catalytic characteristics, such as maximum activity, pH optimum and substrate saturation behavior, in distinct ways. Mg^{2+} supports the highest catalytic activity under most circumstances, which has been observed to be a normal hyperbolic function of either glutamate or ammonia concentration (Segal and Stadtman, 1972). Several different pH optima have been reported for Mg^{2+} enzyme: 8 (Kingdon and Stadtman, 1967), 7.7 (Woolfolk et al., 1966), 9 (Segal and Stadtman, 1972), and 7.5 (Abell and Villafranca, 1991). For Mn^{2+}, lower maximum catalytic activities have been reported, with more acidic pH optima: 7 (Kingdon and Stadtman, 1967), and 6.5 (Abell and Villafranca, 1991). Co^{2+} enzyme exhibits an apparent pH optimum of 7.5, and, unlike Mg^{2+} enzyme, activity is a complex function of ammonia and glutamate concentrations (Segal and Stadtman, 1972).

E. coli glutamine synthetase is regulated *in vivo* by two primary mechanisms: cumulative feedback inhibition and covalent modification (Armstrong, 1983). Cumulative feedback inhibition is affected by eight compounds. Six of these compounds, tryptophan, histidine, glucosamine-6-phosphate, carbamoyl phosphate, CTP and AMP, are metabolic end products of glutamine, and the other two, alanine and glycine, are general indicators of the status of amino acid metabolism. Individually, each of these can only produce

partial inhibition, but together can completely shut down enzyme activity (Mathews and van Holde, 1996).

The second, more interesting regulation mechanism is reversible adenylylation. In this process, an AMP moiety binds to a specific tyrosine residue (thought to be Tyr 397) on one of the enzyme molecule's 12 identical subunits. This tyrosine residue is near the catalytic site, and its adenylylation effectively shuts down catalytic activity within that subunit. The 12 subunits can be independently adenylyated, resulting in a range of possible catalytic activities for each glutamine synthetase molecule (Jiang et al., 1998).

Adenylylation is influenced by the glutamine:α-ketoglutarate ratio: high glutamine favors adenylylation. The process is regulated by a "bicyclic cascade" in which one enzyme, adenylyl transferase, affects adenylylation and deadenylylation based on the activity of another enzyme, uridylyl transferase. Based on the glutamine:α-ketoglutarate ratio, uridylyl transferase either uridylylates or deuridylylates (i.e., adds a UMP moiety to or removes it from) a regulatory protein called P_{II}. This protein then binds to adenylyl transferase; uridylyated P_{II} promotes deadenylylation of glutamine synthetase, whereas deuridylylated P_{II} promotes adenylylation (Engleman and Francis, 1979).

Most of the information about the characteristics of glutamine synthetase presented above has been gathered through observations of the enzyme in its native, or soluble form. However, *in vitro* practical applications rarely incorporate enzymes in this form. Instead, industrial enzymes are typically immobilized, which provides substantial benefits over the use of enzymes in their soluble form.

Enzyme Immobilization

Enzyme immobilization can be defined as restricting an enzyme's mobility by chemical or physical means. This usually entails either the chemical binding of the enzyme to some carrier surface or physically entrapping it within some matrix or enclosure. Many industrial applications employ immobilized enzymes instead of enzymes in their native (soluble) form because immobilization offers these distinct advantages:

Easy separation from solution and reuse: This is probably the most significant benefit offered by immobilization from an industrial standpoint. Soluble enzyme in solution is usually difficult to separate and retrieve, and its reuse is therefore often impractical. In contrast, immobilized enzyme can usually be easily retrieved from solution and reused in future applications (Hartmeier, 1988).

Greater enzyme longevity: Immobilization often improves the operational stability of the enzyme. In particular, improved resistance to high temperatures has been observed (Banerjee, 1993).

Changes in chemical properties: Immobilized enzymes often exhibit different sensitivities to their environment, such as pH optimum, optimal temperature and K_m's for reactants. These changes can be beneficial to overall enzyme activity in some applications.

A classification system for enzyme immobilization methods was first recommended in 1971 at the first Enzyme Engineering Conference. Three of the major immobilization classifications identified were covalent binding, matrix entrapment, and membrane enclosure (Hartmeier, 1988).

In covalent binding methods, a reactive functional group on the enzyme is covalently bound to a reactive group on the surface of some carrier. These carrier surface groups are usually "activated" prior to covalent binding with the enzyme. One widely-used activating agent is cyanogen bromide (CNBr). In a common immobilization method, CNBr is used to activate the hydroxyl groups on the surface of a polysaccharide matrix, such as the agarose derivative Sepharose. CNBr reacts with these hydroxyl groups to form cyanate esters and cyclic imidocarbonates, which in turn bind readily to free amine groups on the enzyme (Hermanson et al., 1992).

Matrix entrapment involves the physical embedding of an enzyme within a porous matrix, usually in gel form. To allow catalytic activity, the pores of the matrix are large enough to allow enzymatic reactants and products to traverse, but too small to allow the enzyme to escape (Hartmeier, 1988). An example of an entrapping matrix is the gel κ-carrageenan. A product of seaweed, κ-carrageenan is water soluble at room temperature, but forms a rigid, porous gel when a cation, typically K^+, is added. Enzyme is often added to soluble carrageenan solution and then extruded drop-wise into a hardening solution to form semi-rigid, enzyme-entrapping beads. Greater rigidity can be provided by treatment of the beads with other hardening reagents such as glutaraldehyde and hexamethylenediamine (Tosa et al., 1979).

Membrane enclosures, or encapsulations, are probably the simplest immobilization methods available. In these methods, soluble enzyme is enclosed within a porous membrane. As in entrapment methods, these pores must be large enough for reactant and

product passage, but too small to allow the enzyme to escape. Dialysis tubing, thin tubing with a controlled, narrow range of pore sizes, is often used for enzyme encapsulation.

Applications of Immobilized Glutamine Synthetase

Review of the literature identified only two published studies involving immobilized glutamine synthetase. In the first study chronologically, published by Tabata and Kido in 1991, immobilized glutamine synthetase was employed to remove ammonia from dialyzed urine samples to prepare them for spectroscopic creatinine measurement. Glutamine synthetase from *Brevibacterium flavum* was immobilized on alkylamine glass beads using glutaraldehyde through Schiff base formation. Enzymatic conversion was carried out in 0.1 M Tris-HCl buffer (pH 7.5), with 7 mM ATP, 21 mM $MgCl_2$ and 50 mM L-glutamate. The study does not report the residual activity (immobilized enzyme activity divided by soluble activity) resulting from this immobilization, nor does it provide the necessary information to calculate it. It does, however, report a pH optimum of 7.0 for both the soluble and immobilized enzyme, and an expanded pH profile for the immobilized enzyme on both the acidic and alkaline sides of this optimum. The immobilized glutamine synthetase also exhibited good operational stability at room temperature, with 80% of its original activity retained after 2 months.

In the second study, Enosawa and coworkers (1996) evaluated immobilized glutamine synthetase as a means of selectively removing ammonia from blood. The enzyme was immobilized on FMP-activated Cellulofine. Ammonia conversions were run

in 72 mM Imidazole-HCl buffer (pH 7.2) with 5 mM ammonia, 4 mM ATP, 30 mM glutamate and 50 mM Mg^{2+}, and yielded 5.2% residual activity.

Project Focus

By catalyzing the conversion of ammonia to glutamine, glutamine synthetase converts a toxic hybridoma cell waste product back into an essential hybridoma cell nutrient. As previously described, Paredes and coworkers (1999) have successfully transfected hybridoma cells with the glutamine synthetase gene, thereby alleviating much of the problem with glutamine depletion and ammonia toxicity in hybridoma cell culture. However, broad application of this approach would require separate transfection of each hybridoma cell line to be cultured. Also, the expression of the glutamine synthetase gene within these transfected cells would likely divert cell metabolic energy from antibody production to cell maintenance, thereby reducing the specific antibody yield. The use of immobilized, extracellular glutamine synthetase within a hybridoma cell cultivation potentially provides the glutamine supplementation and ammonia reduction observed for the gene transfection method, but offers greater flexibility (i.e., incorporation in the culture of any hybridoma cell line), and places no additional cell maintenance burden on the cells.

Although glutamine synthetase is a well known, widely researched enzyme, few attempts have been made to immobilize it or employ it in practical applications. The two applications described above both incorporated covalent-binding immobilization methods. Of the two, only one of the studies, that published by Enosawa and coworkers, reports a residual activity. This result (5.2%, or a loss of 94.8% of soluble activity) is likely

unacceptable for cost-effective use of the enzyme in practical applications. To identify an immobilization method that yields a higher residual activity, further study is necessary. Additional methods within the covalent binding category, as well as entrapment and encapsulation methods, should be investigated, and their effects on glutamine synthetase activity characterized.

Chapter 2 – Objectives and Experimental Approach

The overall objective of this study was to analyze the effectiveness of immobilized glutamine synthetase as a means of extending the "useful life" of a hybridoma cultivation media by reducing the accumulation of toxic ammonia and replenishing the supply of glutamine. To achieve this objective, the following properties of the use of glutamine synthetase were evaluated in a systematic fashion:

1. Activity of soluble glutamine synthetase in buffer;
2. Activity of soluble glutamine synthetase in culture media;
3. Activity of immobilized glutamine synthetase;
4. Hybridoma sensitivity to enzyme reactants; and finally
5. Application of immobilized glutamine synthetase within an active hybridoma cultivation.

The motivation for each of these steps is detailed below.

Soluble Glutamine Synthetase Activity in Buffer

As previously discussed, soluble *E. coli* glutamine synthetase has been previously evaluated under many different experimental conditions. Although such previous studies are informative, the results achieved and conclusions made therein are poorly transferred to the study at hand for several reasons:

Reactant Concentrations: Most published studies, constrained only by solubility limits, have characterized glutamine synthetase activity at relatively high reactant and

cofactor concentrations. One such study, published by Segal and Stadtman in 1972, analyzed enzyme activity in buffers containing up to 100 mM glutamate, and sizeable increases in activity were observed with increasing glutamate. Glutamate concentrations this high are potential inhibitors of hybridoma cell growth. Enzyme activities at non-toxic glutamate concentrations are likely significantly lower.

Buffer Type: Most previous glutamine synthetase activity experiments were performed in Tris buffer, imidazole buffer, or some combination thereof. These buffers have different compositions and buffering capacities than the bicarbonate buffers present in most commercially available mammalian cell culture media, and might therefore affect the enzymatic reaction differently.

Reaction Volume: In most previous studies, enzyme reactions were carried out in spectrophotometer cuvets in volumes as small as 0.2 ml. These volumes are quite different than those used in most bench-scale or industrial-scale bioreactors, and differences in mixing and transport could have a significant effect on enzyme activity.

Experiment Duration: With such small treatment volumes, many of these previous enzyme reactions were observed over the span of minutes. In contrast, enzymatic ammonia conversion within a bioreactor requires activity over an extended period of time.

Enzyme Source: Unlike most earlier studies, in which the enzyme analyzed was harvested from *E. coli* and purified by the researchers, the current study utilizes *E. coli* glutamine synthetase purchased from a commercial supplier. The purity and mean adenylylation state (as described in Chapter 1) of this product is likely different than that

previously characterized. Therefore, there exists a need to characterize enzyme activity at conditions similar to those to be encountered in a culture medium.

This section of the project served as a bridge between previously published studies conducted with relatively broad experimental conditions and the more application specific, constrained conditions required for the current study. Its primary objective was to quantify the effects on enzyme activity of the reactants ammonia, ATP and glutamate, the cofactor magnesium, and the product glutamine so as to determine conditions optimal for enzyme activity within these constraints. To provide the greatest amount of insight into how glutamine synthetase would perform in a cell cultivation for the current study, experiments were carried out in a bicarbonate buffer, with lower substrate concentrations, in larger volumes, and over longer time spans than previously published results.

Soluble Glutamine Synthetase Activity in Culture Media

Although enzymatic activity experiments in a bicarbonate buffer provide a useful characterization, these conditions are not wholly representative of those present in culture medium. Since the desired application of the enzyme in this study involves glutamine and ammonia control within an active hybridoma culture, glutamine synthetase activity must also be characterized in complete culture media, not just its buffer. Culture media is a much more complex solution, containing relatively high concentrations of glucose and glutamine, as well as smaller amounts of other amino acids, vitamins and minerals. The composition of two common culture media is presented in Appendix 1. Most

commercially available media also contain calcium, which, as previously described, is a potent inhibitor of glutamine synthetase activity.

As discussed in the previous chapter, in addition to the substrates provided in standard culture media, mammalian cells also require other nutrients, adhesion proteins, growth factors and cofactors (trace metals), which are often provided by supplementing the media with animal blood serum. The addition of serum further increases the solution complexity, and also adds calcium to the media. Lindl and Bauer (1989), in a thorough compositional analysis of fetal calf serum, identified the Ca^{2+} concentration at approximately 3.4 mM. Serum is notoriously variable in composition, so this value provides only a rough approximation.

The primary objectives of this portion of the study were to characterize the activity of soluble glutamine synthetase in different types of culture media (with and without calcium, with and without serum), and to identify a medium that yields reasonable enzyme activity as well as good hybridoma growth. These objectives were met in two stages. First soluble enzyme activity was characterized in different media treatments with glutamate, ATP, ammonia and Mg^{2+} concentrations identical to one of the treatments from the earlier experiments in bicarbonate buffer. This allowed a direct comparison of activities in buffer and culture media. In the second stage, soluble enzyme activity was analyzed in various culture media conditions with much lower reactant concentrations (5 mM of each and less). This provided information about the rate of the glutamine synthetase forward reaction under more realistic conditions. Such studies also enabled calculation of the equilibrium constant for the reaction.

Immobilization of Glutamine Synthetase

For the application of glutamine synthetase within a cell culture to be cost effective, immobilization of the enzyme is necessary. For the purposes of this study, the key benefit that immobilization provides is reusability. Unlike soluble enzyme, immobilized enzyme can easily be retrieved from solution and used again in future applications.

The two aforementioned studies involving the immobilization of glutamine synthetase either yielded poor residual activity (Enosawa et al., 1996), or did not characterize residual activity (Tabata and Kido, 1991). Therefore, the current study cannot simply reproduce some established immobilization method. Instead, this section of the project evaluated three different immobilization techniques, and quantified the residual enzyme activity offered by each.

Myriad enzyme immobilization techniques have been documented for many different enzymes (Chibata et al., 1978, Laskin, 1985, Uhlig, 1998). Three methods were evaluated in this study, representing the three general immobilization categories of covalent binding, entrapment, and encapsulation. This strategy was followed in order to provide a greater breadth of information about the behavior of glutamine synthetase under very different immobilization conditions. Also, the expectation was that this strategy would provide a better chance of identifying at least one high residual activity-yielding method then, for example, evaluating three covalent binding methods. The three methods evaluated in this study were selected based on the following criteria:

Protocol Availability and Simplicity: Immobilization methods with well documented, simple protocols were sought. In particular, methods applicable to a wide range of enzymes, and with general-use (as opposed to enzyme-specific) protocols were desired.

Cost: Only those methods that did not require high-cost materials or expensive equipment were considered.

Steam-Sterilizability: Only methods utilizing autoclavable materials were considered.

Based on these criteria, the following immobilization methods were chosen:

- Covalent binding to CNBr-activated Sepharose beads
- Entrapment within κ-carrageenan gel beads
- Encapsulation within dialysis tubing

Using published general-use protocols (or slight modifications thereof), glutamine synthetase was immobilized by each of these methods, and the residual activity resulting from each was evaluated.

To allow direct comparisons to soluble enzyme activity, these experiments were conducted in the same bicarbonate buffer with reactant concentrations identical to a previous set of soluble enzyme experiments. The enzyme thermostability resulting from each successful method was also evaluated by observing the degradation of enzyme activity over time while held at 37 °C.

Hybridoma Sensitivity to Enzyme Reactants

The glutamine synthetase-catalyzed conversion of ammonia to glutamine within culture media requires the addition of the reactants glutamate and ATP, and the cofactor Mg^{2+}. Likely the enzyme conversion rate can be maximized at high reactant concentrations, but the amount of reactants that can be added to the media is severely constrained by the sensitivity of the hybridomas to these substrates. Potentially all of the reactants and cofactors (ammonia, glutamate, ATP and Mg^{2+}) could have an inhibitory effect on either cell growth or MAb production. The objective of this section was therefore to evaluate the effects of ammonia, glutamate, ATP and Mg^{2+} on hybridoma cells. These effects were quantified using maximum viable cell density as a metric. Information provided in this experiment helped to identify reactant concentrations that would provide adequate enzyme activity but would not hamper cell growth.

Application of Immobilized Glutamine Synthetase Within an Active Hybridoma Cultivation

With glutamine synthetase activity characterized and the sensitivity of hybridomas to enzyme reactants reasonably understood, the final phase of the project, which applies the enzymatic conversion process to a cell culture, was undertaken. This phase consisted of two stages. First, a bioreactor system incorporating immobilized glutamine synthetase was designed, constructed, and subjected to preliminary testing. Second, the final experiment, which evaluated the effectiveness of immobilized glutamine synthetase as a

means of regulating ammonia and glutamine concentrations within an actual cell culture, was performed.

During the design of the bioreactor system, several key decisions had to be made:

- Which enzyme immobilization method should be used?
- Should the enzyme reaction occur in the hybridoma culture vessel, or in a separate vessel?
- Should the hybridomas be immobilized or cultured in suspension?
- How will enzyme reactants be delivered to the system?
- How much of each reactant should be added, and how often?
- At what ammonia and/or glutamine concentrations should the enzymatic conversion reaction be initiated?
- How will sterility be maintained?

Once these questions had been answered and the system designed and constructed, a "dry run" of the system was performed. This test run did not include cells. Instead, bicarbonate buffer, with ammonia but no glutamine added, was used to simulate spent culture media. Although this represents an oversimplification of actual final-run conditions, it provided a good test of the bioreactor system, as well as additional information about enzyme kinetics at realistic substrate concentrations.

Finally, the glutamine synthetase-incorporating system was applied to an active hybridoma culture. This system was run in parallel with a control system (a simple spinner flask), which was operated similarly to the experimental system, but without an active

enzyme conversion. After initial setup, no external glutamine was added to either system. By observing the ammonia and antibody concentrations of samples taken from each system at identical time points over the cultures' life cycles, the effect of the immobilized glutamine synthetase was quantified. Antibody concentrations attained within the two systems were compared through the use of a commercial enzyme-linked immunoabsorption assay (ELISA).

Chapter 3 – Materials and Methods

All relevant supplies and equipment vendors and part numbers are detailed in Appendix 2.

Soluble Glutamine Synthetase Activity in Buffer

To analyze the effects of varying substrate concentrations on the activity of *E. coli* glutamine synthetase, a five-factor, two-level experiment was performed, with each treatment performed in duplicate. The five factors were the enzymatic reactants ammonia (NH_3), glutamate (GLU), adenosine triphosphate (ATP), the cofactor magnesium (Mg^{2+}), and the reaction product glutamine (GLN). Table 3.1 lists the high- and low-level concentrations for each of the five substrates, and Table 3.2 identifies the concentrations of the various treatments investigated. The experiment was performed in six batches: (1) treatments 1-4, (2) treatments 5-8 and (3) treatments 9-10, each in duplicate.

Solution Preparation

The buffer used for this experiment was composed of 0.4 g/L KCl, 6.4 g/L NaCl, 0.109 g/L NaH_2PO_4, and 3.7 g/L $NaHCO_3$ in deionized water with the pH adjusted to 7.4 with 1N HCl and 1N NaOH. This buffer is identical to that in Dulbecco's Modified Eagle's Medium (DMEM), an effective and popular medium for hybridoma cultivation, and will heretofore be referred to as "DMEM buffer".

Substrate	NH_3	Glu	ATP	Mg^{2+}	Gln
High Level (mM)	20	20	20	30	5
Low Level (mM)	10	10	10	15	0

Table 3.1 Soluble enzyme experiment – high and low substrate levels.

Substrate/ Treatment	NH_3 Conc. (mM)	Glu Conc. (mM)	ATP Conc. (mM)	Mg^{2+} Conc. (mM)	Gln Conc. (mM)
1	20	20	20	30	0
2	20	10	10	30	0
3	20	20	10	15	0
4	20	10	20	15	0
5	20	10	20	15	5
6	10	10	20	15	0
7	20	10	10	15	0
8	10	10	10	15	5
9	20	20	20	15	0
10	10	10	20	15	5

Table 3.2 Soluble enzyme experiment – treatment concentrations.

The following procedure was followed to prepare each treatment:

1. The desired mass of each of the five substrates in solid form (ammonium chloride for ammonia, L-glutamic acid for glutamate, ATP disodium salt for ATP, magnesium chloride hexahydrate for magnesium, and L-glutamine for glutamine), was weighed and added to 14 ml of DMEM buffer in a small beaker with a magnetic stir bar on a stir plate, and completely dissolved.
2. 200 μL of penicillin-streptomycin solution was added to prevent microbial growth, and the solution diluted to 20 ml with additional DMEM buffer in a graduated cylinder.
3. The pH of the solution (which dropped dramatically upon the dissolution of ATP and glutamic acid) was adjusted to 7.4 with 0.2 N HCl and 0.2 N NaOH.
4. 2.5 ml was removed and immediately frozen in a 15 ml centrifuge tube as an initial sample.
5. 10 units of *E. coli* glutamine synthetase in lyophilized powder form was dissolved in 1 ml DMEM buffer (by definition, one unit is enough enzyme to convert 1.0 μmole of L-glutamate to L-glutamine in 15 minutes at pH 7.1 at 37 °C). This soluble enzyme was then added to the treatment solution.
6. The solution was sterilized via filtration through a 0.2 μm syringe filter into a sterile 50 ml centrifuge tube within a sterile laminar-flow hood. It was then sealed and placed in the experimental environment: an incubator at 37 °C and 5% CO_2, with no stirring.

All solid reagents, the glutamine synthetase powder, and the penicillin-streptomycin solution were purchased from Sigma (St. Louis, MO).

Sample Collection

Five samples (in addition to the initial sample) were removed from each treatment solution over the course of between sixty and seventy hours. No additions to the treatments were made, except for pH adjustments, as described below. The elapsed time between samples varied from batch to batch, but sampling of all batches was performed more frequently during the early stages of the experiment, so as to adequately follow the fastest stage of the reaction. To prevent bacterial or fungal contamination, all sampling was done inside a laminar-flow hood using sterile equipment. Each treatment solution was first mixed briefly by inversion, and 2.5 ml of solution was then removed with a sterile pipette and transferred to a 15 ml centrifuge tube.

As previously described, the forward reaction catalyzed by glutamine synthetase yields one proton per glutamine molecule produced. Therefore, the pH of the treatment solutions usually dropped below 7.4 between sampling intervals, which, as described earlier, can adversely affect the reaction rate. To quantify this pH change, the pH of each 2.5 ml sample was read via a standard pH electrode and adjusted back to 7.4 with 0.2 N NaOH. The volume of NaOH required to affect this pH change was recorded, and then multiplied by the volume in ml of treatment solution remaining after the current sample was removed divided by 2.5 ml, which identified the volume of NaOH needed to return the treatment solution to pH 7.4. This volume of sterile-filtered 0.2 N NaOH, or a molar-

equivalent volume of 1 N NaOH, was then added via sterile pipette to the treatment solution inside a laminar-flow hood, and the treatment solutions re-capped and returned to the incubator. After pH adjustment, the sample tubes were re-capped and frozen at -5 °C until the collection of the last samples of that batch, when all samples were analyzed. The drop in temperature was assumed to quench further reaction.

Sample Analysis

Since ammonia control is the primary objective of this study, glutamine synthetase activity was characterized by quantifying the solution ammonia concentration over time. Ammonia concentrations were read with an ammonia-selective electrode manufactured by Orion (Beverly, MA). This electrode utilizes a gas-permeable membrane at its tip, through which gaseous ammonia passes and reacts with a filling solution within the electrode casing. The ensuing reaction affects a pH change which is measured by an internal pH electrode. The resulting output is measured in millivolts, which provides a linear correlation with ln(ammonia concentration) (Orion Catalog, 1999).

Before taking ammonia measurements of a batch of samples, a three-point calibration of the ammonia electrode was performed. Using a 100 mM NH_4Cl standard solution and deionized water, 2 ml standard solutions of 30 mM, 10 mM and 1mM ammonia were freshly prepared prior to each set of measurements. 40 μL (or 2% of the solution volume) of an ionic strength adjusting (ISA) solution, also from Orion, was added to each standard solution to adjust its pH to between 11 and 13, the required range for accurate electrode readings. To take readings of each calibration standard, the tip of the

ammonia electrode was submerged in the solution and tapped to remove any air bubbles adhering to the membrane. To allow equilibration within the electrode, eight minutes was allowed to elapse before a final voltage reading from a combination pH/voltage meter was read and recorded. Before transferring the electrode to another solution, the lower casing and tip of the probe were thoroughly rinsed with deionized water and patted dry. From outputs generated from all three ammonia standards, a calibration curve was produced. A sample calibration curve for this electrode is presented in Appendix 3. The error associated with ammonia measurement depends on the absolute concentration of ammonia, as illustrated in Figure 3.1. As this figure indicates, 1 mV reading errors at ammonia concentrations of 10 mM and 20 mM correspond to approximately 0.38 mM and 0.77 mM ammonia concentration measurement errors, respectively.

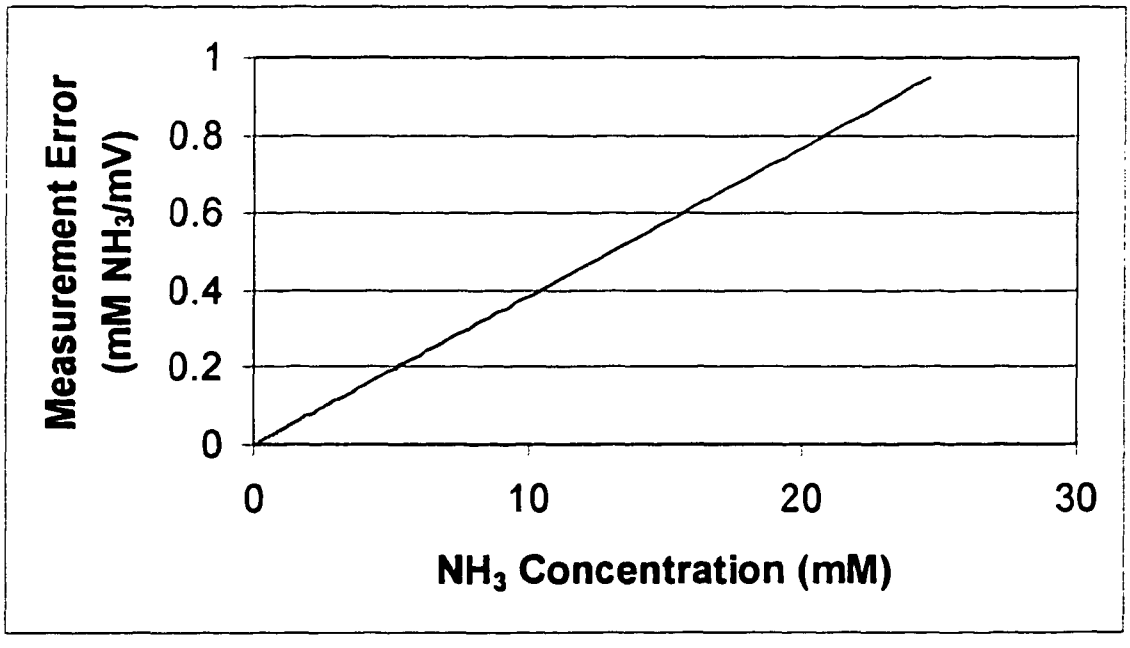

Figure 3.1 Ammonia-sensing electrode – representative measurement error curve.

When a satisfactory calibration curve had been generated, the experimental samples were thawed and read in the same manner as the calibration standards. Only four samples were thawed at a time to prevent further enzyme reaction during the time between thawing and ammonia reading.

ATP Hydrolysis

As described previously, the pH of each experimental solution dropped dramatically upon the addition and dissolution of ATP. Since the ATP used in all experiments was in disodium salt form, this pH drop suggested that a substantial amount of the added ATP was undergoing spontaneous hydrolysis, a process which releases one proton per ATP molecule degraded, upon dissolution. Since the ADP^{3-} form of ADP is greatly favored over the ADP^{2-} form in solutions of pH 7.0 and above (Mathews and van Holde, 1996), the majority of this hydrolysis likely proceeded according to the equation

$$ATP^{4-} + H_2O \Leftrightarrow ADP^{3-} + HPO4^{2-} + H^+.$$

Since ADP cannot substitute for ATP as an energy source in glutamine synthetase-catalyzed ammonia conversion, this hydrolysis reduced the amount of ammonia that could be converted when glutamate and Mg^{2+} were abundant. In addition, Mg^{2+} readily complexes with ATP in solution and is known to introduce a strain into the ATP molecule that promotes hydrolysis (Mathews and van Holde, 1996).

To quantify the extent of spontaneous ATP hydrolysis upon dissolution, as well as the effect of Mg^{2+} on the process, a degredation experiment was performed. In this experiment, 1 liter of ultrafiltered deionized water was titrated with 1N NaOH, and a

calibration titration curve generated. Eight experimental treatments were then prepared by dissolving varying amounts of ATP and $MgCl_2$ in 10 ml of deionized water. These solutions were then also used to titrate ultrafiltered deionized water. By comparing pH changes for each experimental titration to the calibration curve, the concentration of H^+ ions released per volume of ATP solution added was identified. Due to the 1:1 ratio of H^+ and ADP produced in the hydrolysis reaction provided above, these results allowed indirect measurement of the extent of ATP hydrolysis for each of the eight treatments, which are listed in Table 3.3.

Treatment	ATP Concentration (mM)	Mg^{2+} Concentration (mM)	Titration Receiving Volume (ml)
1	30	30	30
2	20	30	20
3	20	20	20
4	20	15	20
5	20	0	20
6	10	15	10
7	10	10	10
8	2	2	2

Table 3.3 ATP hydrolysis experiment: solution concentrations and titration receiving volumes.

Soluble Glutamine Synthetase Activity in Culture Media

Glutamine synthetase activity was analyzed under several different substrate concentration conditions in two different culture media: Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium - Eagle for Suspension Cultures (S-MEM), both purchased in powder form from Sigma. The components list and preparation protocol provided by Sigma for each is presented in Appendix 1.

Solution Preparation

Each of the two types of culture media were prepared according to the Sigma-recommended protocol, with the exception that 0.292 g/L of L-glutamine and 0.802 g/L of glucose, each in solid form, were added to the S-MEM. These additions brought the total glutamine concentration to 4 mM (the same as DMEM) and the glucose concentration to 10 mM. Both types of media were prepared in one-liter batches, sterile filtered upon final dilution and pH adjustment, and refrigerated between uses. For those experimental treatments which were performed in media plus 10% serum, sterile horse serum (Sigma), which had been previously heat-inactivated at 56 °C for 30 minutes, was added.

With culture media replacing buffer solution, experimental treatment solutions were prepared using the same protocol as described for the buffer experiment, with the following exceptions:

1. Instead of glutamic acid, solid monosodium glutamate was used as a glutamate source; this change was made to eliminate the acidification by glutamic acid.
2. Instead of penicillin/streptomycin solution, an equal volume of antibiotic/antimycotic solution (Sigma), which includes the antifungal amphotericin B in addition to penicillin and streptomycin, was used. This solution has a useful life at 37 °C of only three days (Sigma Catalog, 1999), so additional doses were added as necessary.
3. The treatment solutions were not sterile filtered; instead the antibiotic/antimycotic solution was relied upon to control microbial growth.

Sample Collection

The use of antibiotic/antimycotic solution allowed a simpler sample collection procedure than that used for the buffer experiment. At the desired sampling time, the entire treatment solution was removed from the incubator, and its pH read non-sterilely and adjusted back to 7.4 with 0.2 N NaOH. After this bulk pH adjustment, a 1.5 ml sample was transferred to a 15 ml centrifuge tube and immediately frozen for batch analysis upon the experiment's completion.

Sample Analysis

Ammonia concentration readings of these culture media samples were initially attempted in the same manner used for buffer samples: by submerging the ammonia electrode tip in the solution. However, surfactants in the media quickly caused the

electrode's hydrophobic membrane to become wet, thus yielding poor results and requiring frequent membrane replacement. Therefore, culture media ammonia readings were performed by suspending the electrode tip approximately 2 mm above the sample surface within its centrifuge tube. The gap at the top of the tube opening between the tube and the electrode casing was sealed with parafilm, and the system allowed to equilibrate for eight minutes before the voltage output was recorded. This scheme was a small-volume modification of a protocol recommended by the probe manufacturer for surfactant-containing samples. Accurate measurements were obtained through this approach.

Immobilization of Glutamine Synthetase

Covalent Binding to CNBr-activated Sepharose Beads

CNBr-activated Sepharose 6% agarose macrobeads in lyophilized powder form were purchased from Sigma and used for all treatments of this experiment. To couple the soluble *E. coli* glutamine synthetase to these beads, two different protocols were followed, and the resulting enzymatic activity of each compared. Protocols employed were slight modifications of published procedures, and will be referred to here as 1) the Modified Sigma protocol (1996), and 2) the Modified Hermanson protocol (Hermanson et al., 1992). These two protocols are generally similar, but with some practical differences which impact enzyme activity.

The Modified Sigma Protocol

1. 50 units of lyophilized *E. coli* glutamine synthetase were dissolved in 1.4 ml of buffer A, consisting of 0.1 M $NaHCO_3$ and 0.5 M NaCl in deionized water with a pH of 8.5.
2. 0.2 g of dry sepharose beads were soaked in 10 ml of cold 1mM HCl for 30 minutes. The HCl was then removed via suction funneling through qualitative filter paper. Two to three additional washings of 20 ml HCl were passed through the funnel. These washings are necessary to remove lactose from the sepharose beads, which is added during the freeze-drying process.
3. The beads were then rinsed with several aliquots of deionized water, and then with Buffer A.
4. After partial funnel-drying, the beads were immediately removed from the filter paper with a small spatula and transferred to a 20 ml scintillation vial containing the soluble glutamine synthetase.
5. The sepharose bead/enzyme mixture-containing vial was then sealed, attached upright to a rotating shaker plate, and mixed for two hours at room temperature to allow coupling of the enzyme to the beads.
6. After coupling, the beads were transferred back to a suction filter and washed several times with Buffer A.
7. To block any unreacted groups which may remain on the beads, the beads were transferred to a scintillation vial with 10 ml of 1 M ethanolamine, pH 8.0, and incubated at room temperature for two hours with no additional mixing.

8. When blocking was complete, the beads were transferred to a suction filter, and the ethanolamine rinsed away with five washing cycles of Buffer A, followed by Buffer B, which contained 0.1 M acetate/0.5 M NaCl at pH 4.
9. After thorough rinsing, the beads were gently scraped from the filter paper and stored at 4 °C in 10 ml of 1.0 M NaCl and 100 microliters of penicillin/streptomycin solution until use.

The Modified Hermanson Protocol

This protocol follows the above protocol, but with the following deviations:

1. In step 5, the sepharose bead/enzyme mixture was mixed on the rotating plate for 24 hours at 4 °C.
2. In step 7, blocking of unreacted groups was performed in 1.0 M ethanolamine, pH 9.0 on a rotating plate for one hour at room temperature.
3. In step 8, Buffer C, 1 M NaCl, pH 7, was substituted for Buffer B.

The most significant difference between these two protocols is that, in the Sigma protocol, the immobilized enzyme is washed with a highly acidic (pH 4.0) solution, whereas in the Hermanson protocol, the enzyme always contacts solutions with pH's close to physiological levels.

All experiments quantifying the activity of sepharose bead-immobilized glutamine synthetase were performed non-sterilely in DMEM buffer with 1% antibiotic/antimycotic solution. The beads and the treatment solution were placed in a sealed 20 ml scintillation vial, and gently mixed on a rotating plate in an incubator at 37 °C. At the desired

sampling time, the vial was removed from the incubator and the beads allowed to settle to the bottom. The pH within the vial was then read and adjusted back to 7.4 with 0.2 N NaOH. After pH adjustment, a 1.5 ml sample was removed and frozen for batch processing at a later date (no more than a week). Ammonia readings were performed in the same manner as in the soluble-enzyme-in-buffer experiment.

Entrapment Within κ-Carrageenan Gel Beads

κ-carrageenan powder purchased from Sigma was used in varying concentrations to form glutamine synthetase-entrapping gel beads. The resulting bead characteristics and enzyme activities were observed in both DMEM buffer and DMEM media. The beads were prepared via the following protocol:

1. 2 ml of deionized water was sealed in a 15 ml centrifuge tube and heated to 50 °C in a water bath.
2. The desired mass of κ-carrageenan powder was weighed and added to the deionized water at the desired concentration, and this solution mixed on a test-tube vortexer and with vigorous stirring with a small spatula until homogeneous.
3. Glutamine synthetase dissolved in deionized water was pipetted into a microcentrifuge tube and heated to 37 °C in a water bath.

4. The glutamine synthetase solution was added to the centrifuge tube containing the carrageenan gel, and thoroughly mixed via vortexing and mechanical stirring.
5. The enzyme-containing gel was quickly transferred to a 3 ml disposable syringe, and extruded drop-wise into a stirred beaker of hardening solution containing either 0.3 M $MgCl_2$ or 0.3 M KCl. During extrusion, the syringe was rapidly tapped with a finger to reduce the drop size.
6. The beads were refrigerated in the hardening solution for one hour, rinsed with deionized water on a suction funnel, and then immediately used experimentally.

All experimental treatments involving κ-carrageenan beads were performed non-sterilely with antibiotic/antimycotic solution. Carrageenan beads and treatment solutions were placed in 20 ml scintillation vials and mixed lightly on a rotating plate in an incubator at 37 °C. At the desired sampling time, pH's were read and adjusted and samples collected as described in the sepharose-bead section. Ammonia readings were performed by electrode tip submersion for buffer samples, and by suspension over the sample surface for media samples.

Encapsulation Within Dialysis Tubing

Spectra/Por® dialysis tubing with a molecular weight cut-off between 12,000 and 14,000 and Spectra/Por® closures were purchased from VWR (Phoenix, AZ). The activity of glutamine synthetase immobilized within this tubing was tested in both DMEM

buffer and S-MEM media with 10% horse serum. Initial experiments involving dialysis tubing immobilization were performed non-sterilely with antibiotic/antimycotic solution to prevent contamination. The final phase of this project, the application of immobilized glutamine synthetase to an active hybridoma culture, required sterile encapsulation of the enzyme; this protocol will be described later.

For the initial dialysis tubing experiments, soluble glutamine synthetase in DMEM buffer was pipetted into an approximately four-inch length of wet dialysis tubing, and the ends were sealed with Spectra/Por® closures. The filled tubing was then immediately submerged in a 50 ml centrifuge tube containing the appropriate treatment solution, and transferred to the experimental conditions: a rotating plate in an incubator at 37 °C. Samples were collected and analyzed and pH adjusted in the same manner as described in the κ-carrageenan immobilization experiment.

Hybridoma Sensitivity to Enzyme Reactants

To analyze the sensitivity of hybridoma cells to the reactants and cofactor of the glutamine synthetase forward reaction, a four-factor, two-level experiment was performed in S-MEM plus 10% horse serum. The four factors analyzed were ammonia, glutamate, ATP and magnesium. Table 3.4 lists the high- and low-level concentrations for each of the five substrates, and Table 3.5 identifies the initial concentrations of the various treatments investigated.

Substrate	NH_3	Glu	ATP	Mg^{2+}
High Level (mM)	3	10	10	8
Low Level (mM)	0	0	0	0

Table 3.4 Cell sensitivity experiment – high and low substrate levels.

Substrate/ Treatment	NH_3 Conc. (mM)	Mg^{2+} Conc. (mM)	Glu Conc. (mM)	ATP Conc. (mM)
1	0	0	0	0
2	0	8	10	0
3	3	8	0	0
4	3	0	10	0
5	0	8	0	10
6	0	0	10	10
7	3	0	0	10
8	3	8	10	10

Table 3.5 Cell sensitivity experiment – treatment concentrations.

Murine-murine hybridoma cells, ATCC #CE9H9, were used exclusively in this study. These cells produce IgG_1-type monoclonal antibodies specific to insulin from human, cow, pig and rabbit, and to pre-insulin from cow and pig (ATCC, 1999). To prepare the cells for this experiment, they were transferred from DMEM plus 10% serum,

their recommended culture media, to S-MEM plus 10% serum in an 80 cm^2 tissue culture flask. The cells were allowed to adapt to S-MEM over the course of one week, and their cell density maintained between $1x10^5$ and $1x10^6$ cells/ml during this period via subculturing with new S-MEM as necessary. In general, these cells replicate every 24 hours, so fresh medium is required every 2 days at a dilution of 1:4.

Solution Preparation

64 ml of previously prepared S-MEM plus 10% horse serum was split into eight 8 ml aliquots in 15 ml centrifuge tubes. As in the soluble enzyme experiments, reagents were added to solution in the desired amounts in their solid form, dissolved completely, and the resulting solution pH's adjusted to 7.4 with 1 N NaOH. These eight treatment solutions were then transferred into a laminar-flow hood and sterilized through a 0.2 μm syringe filter directly into 25 cm^2 tissue culture flasks.

Eight equal aliquots of S-MEM-adapted cells were pipetted into sterile 15 ml centrifuge tubes, and centrifuged at 1000 rpm for ten minutes to separate the cells from the medium. The aliquot volume used was calculated based on a desired initial cell density of $1x10^5$ cells/ml within each 8 ml experimental treatment. After centrifugation, the cell-containing tubes were transferred to a laminar-flow hood, the supernatant within each decanted, and the cells in each transferred to one of the eight experimental 25 cm^2 flasks. The eight flasks were then sealed with gas-permeable caps and transferred to a cell-culture incubator maintained at 37 °C and 5% CO_2. The flasks were treated as a pure batch system: no subsequent media replenishment or pH adjustment was performed.

Sample Collection and Analysis

Samples from each of the eight treatments were taken together, usually twice a day, over the span of five days. At the desired sampling time, the flasks were removed from the incubator and a 0.2 ml sample from each was sterile pipetted into small test tubes. These 0.2 ml samples were stained with an equal volume of 0.4% trypan blue solution, and cell counts were taken for each on a hemocytometer under a light microscope. Counts of both living (unstained) and dead (stained) cells were recorded, and viable cell densities calculated for each according to the formula:

Cell Density [cells/ml] = (living cell count / # of hemocytometer boxes counted) * 20,000,

where the factor 20,000 accounts for dilution with trypan blue solution and the volume of the counting chamber.

Immobilized Glutamine Synthetase in an Active Hybridoma Cultivation

Bioreactor System Design

The bioreactor system as designed and constructed is represented in Figure 3.2, and its components identified and described in Table 3.6. Within an incubator maintained at 37 °C and 5% CO_2 were housed two vessels: a 500 ml working volume spinner flask, and a 100 ml working volume spinner flask. The larger flask was the hybridoma culture flask, in which the cells and the bulk of the media were contained. The smaller flask, or "reaction chamber", contained soluble glutamine synthetase encapsulated within dialysis

tubing. Both of these vessels rested on a magnetic stir plate; however, to avoid damage to the dialysis tubing, the paddle stirrer was removed from the reaction chamber vessel. In its place, a small 0.25 inch diameter cross-shaped magnetic stirrer was added to this vessel, which provided light mixing.

The two flasks inside the incubator were connected by flexible tubing, allowing fully enclosed transfer of spent media from the culture flask to the reaction chamber, and transfer of glutamine-refreshed media back to the culture flask. Due to space constraints inside the incubator, the tubing connecting the two vessels passed outside of the incubator, where a reversible pump affected fluid transfer. Also on this inter-vessel line was a sampling port, consisting of two three-way valves, a 3 cm^3 syringe and a small piece of tubing. This port allowed sampling of either culture-flask or reaction-chamber media, and subsequent sterilization of the port with 70% ethanol solution.

To allow enzymatic ammonia conversion, the bioreactor system also allowed flow of soluble glutamate, ATP and Mg^{2+} directly to the reaction chamber. Reservoirs containing concentrated solutions of these substrates were stored outside the incubator, and were connected to the reaction chamber via flexible tubing. Since Mg^{2+} readily complexes with ATP in solution, and affects a conformational change within the ATP molecule that encourages spontaneous hydrolysis (Mathews and van Holde, 1996), these compounds were kept separated until addition to the reaction chamber. One reservoir contained concentrated Mg^{2+}, and the other contained concentrated ATP and glutamate.

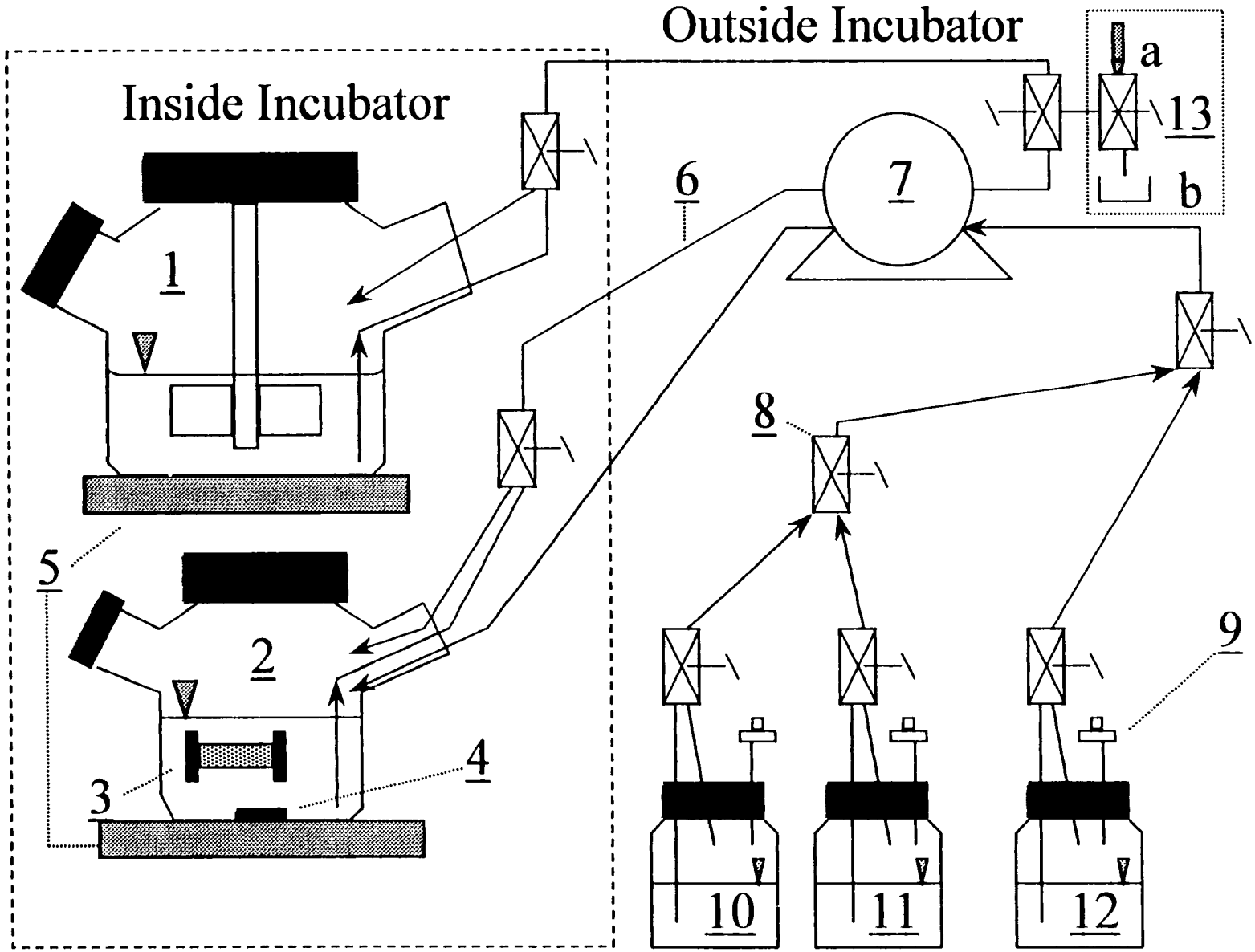

Figure 3.2 Bioreactor System Schematic.

Code	Component	Equipment/Materials Description
1	Hybridoma Culture Chamber	500 ml working volume spinner flask
2	Enzyme Reaction Chamber	100 ml working volume spinner flask
3	Immobilized Enzyme	Soluble glutamine synthetase in dialysis tubing
4	Reaction Chamber Mixer	Cross-shaped magnetic stir bar - 0.25 in. diameter
5	Stir Plate	Variable-speed, five-position magnetic stirrer
6	Flexible Tubing	2.79 mm ID Tygon Tubing
7	Pump	Reversible, dual-channel, 10 to 60 rpm pump
8	Valves	Three-way valves w/ luer-lock connections
9	Air Vent	0.2 micron filter w/ luer-lock connection
10	1N NaOH Reservoir	125 ml media bottle w/screw cap
11	$MgCl_2$ Reservoir	125 ml media bottle w/screw cap
12	ATP, Glutamate, Glucose Reservoir	125 ml media bottle w/screw cap
13	Sampling Port	
a)		3cc disposable syringe w/ luer-lock connection
b)		70% ethanol solution

Table 3.6 *Bioreactor system component descriptions.*

Also, since the glutamine synthetase forward reaction and spontaneous ATP hydrolysis both release H^+, a third reservoir containing 1N NaOH was included in the system to regulate media pH. Based on media pH and ammonia concentration readings, solutions from each of these three reservoirs could be pumped into the reaction chamber in any volume or ratio.

Bioreactor System -- Test Run

To provide a test of the effectiveness and integrity of the bioreactor system, a preliminary test run was performed. As previously discussed, this test did not involve the use of cells or culture media. Instead, the buffer of S-MEM media (heretofore referred to as "S-MEM buffer"), with ammonia added to simulate spent culture media, was used as the test solution. To further simplify the test, it was not performed sterilely, but

antimycotic/antibiotic solution was employed to inhibit microbial growth. To quantify the activity of the dialysis tubing-entrapped glutamine synthetase, buffer samples were collected and their ammonia concentrations immediately measured.

Experiment Set-Up

After assembly of the bioreactor system equipment, the following steps were performed:

1. S-MEM buffer was prepared. This buffer consisted of 0.4 g/L KCl, 6.8 g/L NaCl, 1.22 g/L NaH_2PO_4, 2.2 g/L $NaHCO_3$, and 0.011 g/L phenol red in filtered deionized water, as well as 1% antibiotic/antimycotic solution. The pH was adjusted to 7.4 with 1N NaOH and 1N HCl. To 150 ml of this buffer was added 4mM NH_4Cl to simulate the approximate ammonia level in completely glutamine-depleted S-MEM media. 130 ml of this 150 ml was then transferred to the bioreactor system culture flask, and the remaining 20 ml to the reaction chamber.
2. Concentrated reactant solutions were prepared. Two separate solutions, 20 ml of 80mM ATP and 80mM monosodium glutamate, and 10 ml of 400 mM $MgCl_2$, were each prepared in S-MEM buffer and transferred to their respective bioreactor system reservoir bottles. 20 ml of 1N NaOH was also transferred to its reservoir bottle at this time.
3. *E. coli* glutamine synthetase was dissolved and immobilized. 100 units of enzyme in powder form was dissolved in 2 ml of S-MEM buffer, and

encapsulated within dialysis tubing using the same materials and protocol as detailed earlier. When completed, this enzyme "packet" was placed inside the bioreactor system reaction chamber.

4. The enzyme reaction was initiated. Approximately 0.62 ml of Mg^{2+} solution and 3.12 ml of glutamate/ATP solution were pumped into the reaction chamber. These volumes were measured via markings on the flexible tubing at 0.25 ml increments. Additional buffer from the culture flask was then pumped into the reaction chamber until the total liquid volume in the reaction chamber reached 50 ml. With these volumes, the initial Mg^{2+}, ATP and glutamate concentrations were all 5 mM. Finally, the magnetic stir plate under both flasks inside the incubator was switched on.

Sample Collection and Analysis

At each desired sampling time, the following steps were taken:

1. The magnetic stir plate was switched off, and the solutions inside the two flasks allowed to sit unmixed for five minutes. (Although this "settling period" was not necessary for this trial run with no cells, it provided a better simulation of the expected final-run protocol.)
2. Buffer from the reaction chamber was sampled. To collect each sample, buffer was pumped from the reaction chamber to the sampling port, and 2 ml was collected in a 15 ml centrifuge tube. The buffer remaining in the tubing

was pumped back to the reaction chamber, and the magnetic stir plate switched on.

3. The pH of the sample was measured and adjusted back to 7.4 with 0.2 N and/or 1 N NaOH. The volume of NaOH required to provide this adjustment within the 2 ml sample was recorded, and was then scaled up, by multiplying it by 25, to the volume necessary to adjust the pH of the entire reaction chamber volume to 7.4.
4. The ammonia concentration of the sample was measured with an ammonia-sensing electrode using the method described earlier for culture media samples. The voltage output from the electrode was converted to mM using a standard curve from a two-point (1 mM and 10 mM ammonia) calibration.
5. If the desired ammonia conversion within the reaction chamber was complete, the buffer from the reaction chamber was pumped to the culture flask. To avoid drying out of the dialysis tubing inside the reaction chamber, the new batch of buffer from the culture flask, and then the reactant solutions and NaOH from their reservoirs, were immediately pumped into the reaction chamber.

Bioreactor System – Final Run

The final run was expected to use the same equipment and sampling protocols as the test run described above, but with an active hybridoma culture in sterile media instead of non-sterile buffer. However, when the bioreactor system was autoclaved, the three-

way valves on both the media-transfer and reactant-addition lines were damaged. The fit between the moving and stationary portions of the valves became loose, and subsequent attempts to pump fluids through tubing incorporating them were ineffective.

A new experimental system and sampling protocol were therefore developed. Under this modified system, the culture flask and reaction chamber were not interconnected via tubing, nor were reactants pumped to the reaction chamber. Instead, the two flasks functioned as stand-alone vessels, and all media and reactant solution transfers were performed inside a laminar-flow hood via pipette. Due to the suspected compromise of sterility caused by the valve failure, antibiotic/antimycotic solution was used liberally to inhibit microbial contamination.

To allow evaluation of the effect of glutamine synthetase on the hybridoma culture, a control system which did not include the enzyme was necessary. In this experiment, the control system was a simple spinner flask, similar to the culture flask of the experimental system. Both systems used the same media and serum concentration, and care was taken to treat the experimental and control flasks identically in terms of pH control, atmospheric O_2 and CO_2, temperature, fluid volume and initial cell density. All samples from the two flasks were both removed and measured within minutes of each other, and reactant solution additions to the experimental flask were matched with additions of equal volumes of buffer to the control.

Within both the experimental and control culture flasks, hybridomas were immobilized in agarose gel. Immobilization of the cells allowed a greater cell density than would a suspension culture, which was desired to shorten the time required to reach

medium glutamine exhaustion, and therefore shorten the experiment. Also, it was feared that suspension cells would clog the pores on the dialysis tubing encapsulating the glutamine synthetase in the reaction chamber; cell immobilization was anticipated to reduce this adverse effect.

Like glutamine, glucose within the media of both systems would have been rapidly depleted without supplementation. There was concern that the effect of limiting glucose would mask or overwhelm any differences between the experimental and control systems. To avoid this difficulty, both systems were periodically supplemented with equal volumes of a high-concentration glucose solution.

As in the test run, the pH and ammonia concentration of each sample was immediately measured, which provided important feedback regarding the enzymatic conversion progress. However, the key metric for evaluating the effect of an enhancement to a hybridoma culture is monoclonal antibody production. Therefore, a portion of some of the samples from both the experimental and control flasks were frozen, and their relative monoclonal antibody concentrations later measured using an ELISA enzyme immunoassay.

Cell Immobilization in Agarose Gel

Hybridomas were entrapped using a modified version of a protocol described by Nilsson and coworkers (1986). 80 ml of 2% (w/v) beads (40 ml each for the experimental and control flasks) with one million viable cells/ml beads were desired. The following procedure was followed:

1. 1.6 g of Type IX-A agarose powder (Sigma, St. Louis, Mo) was added to phosphate-buffered saline (PBS), pH 7.4 to a total volume of 40 ml, and then dissolved completely at 70 °C. The resulting solution contained 4% (w/v) agarose. Type IX-A is an ultra-low gelling temperature agarose which yields a gel with relatively strong mechanical properties.
2. The agarose solution, and another beaker containing 100 ml of mineral oil (Walgreens), were autoclaved at 120 °C for 20 minutes, and then stored in an incubator at 37 °C for later use.
3. 120 ml of S-MEM culture media containing 6.8×10^5 viable hybridoma cells/ml was centrifuged for 10 minutes at 1000 rpm in three 50 ml centrifuge tubes, and the supernatant media decanted. The cells were then redissolved in 40 ml of fresh, sterile S-MEM media, pH 7.4 with 4 mM glutamine, 10 mM glucose, and 10% horse serum.
4. The cell-containing media was added to the 4% agarose solution and mixed thoroughly at 37 °C.
5. The media-agarose solution was added to the sterile mineral oil and mixed vigorously with a magnetic stir bar on a stir plate. Mixing was stopped when uniform agarose beads of approximately 300 μm diameter were produced.
6. The mineral oil-agarose bead emulsion was transferred to an ice bath for five minutes, 50 ml of S-MEM was added to allow separation of the hydrophilic agarose beads from the oil, and the entire solution allowed to sit at room temperature for 10 minutes.

7. The solution was poured into several sterile centrifuge tubes and centrifuged for 10 minutes at 1000 rpm.
8. The mineral oil was removed via sterile pipette from the surface of each centrifuge tube, and the contents of all the tubes consolidated in a sterile 125 ml media bottle, and divided into two equal volumes with 40 ml of beads.

While this procedure was successful in encapsulating cells, there was some cell leakage out of the beads. These suspension cells were undesired but later provided the opportunity for monitoring cell growth.

Experiment Set-Up

260 ml of fresh, sterile S-MEM with 4 mM glutamine, 10 mM glucose and 10% horse serum was added to both the control and experimental flasks. The agarose beads were then transferred to each flask, and the flasks placed in an incubator at 37 °C and 5% CO_2.

Both spinner flasks were cultured as batch systems until their ammonia concentrations reached 3 mM. At this point, the reaction chamber of the experimental system was prepared according to the following procedure:

1. The reaction chamber containing a 0.25 inch cross-shaped stir bar, and a 4 inch piece of dialysis tubing with closures were autoclaved at 120 °C for 20 minutes.

2. 200 units of *E. coli* glutamine synthetase were dissolved in S-MEM buffer, sterilized through a 0.2 μm syringe filter, and encapsulated in the sterile dialysis tubing.
3. Three solutions were prepared in S-MEM buffer, adjusted to pH 7.4 with 1N NaOH and filtered at 0.2 μm: 50 ml of 100 mM ATP, 100 mM monosodium glutamate and 200 mM glucose, 20 ml of 500 mM $MgCl_2$, and 50 ml of 200 mM glucose. These solutions, as well as 20 ml of sterile-filtered 1 N NaOH, were stored in sterile centrifuge tubes.
4. The encapsulated enzyme, 5 ml of the ATP/glutamate solution, 1 ml of the Mg^{2+} solution, and 94 ml of media from the experimental culture flask were sterilely transferred to the reaction chamber. This vessel was moved to the stir plate inside the incubator.

Sample Collection and Analysis

At the desired sampling time (usually twice a day), the experimental and control culture flasks and the reaction chamber were moved from the incubator to a laminar-flow hood, and the agarose beads allowed to settle for 5 minutes. 1.5 ml, 1.6 ml and 3.1 ml were then sterilely pipetted from the reaction chamber, the experimental culture flask, and the control culture flask, respectively, to sterile 15 ml centrifuge tubes. At one of the sampling times each day, an additional 0.5 ml was removed from the two cell culture flasks, and frozen for later use in the enzyme immunoassay. 0.1 ml from each of the two culture flask samples was mixed with 0.1 ml 0.4% trypan blue solution and suspension

cell counts performed as described earlier. 1.5 ml from each of the three samples was then used for pH and ammonia measurement, using the same methods described for test-run samples.

If ammonia conversion within the current reaction chamber media batch was complete, this media was temporarily transferred to a sterile media bottle, and sterile 1 N NaOH added to raise its pH to 7.4. Media from the experimental culture flask and reactant solution were then immediately pipetted into the reaction chamber to a total fluid volume of 100 ml, and the media in the sterile media bottle cycled back to the experimental culture flask. Finally, a volume of the sterile concentrated glucose solution equal to volume of reactant solution added to the reaction chamber was pipetted into the control flask, and all three flasks returned to the stir plate in the incubator.

Monoclonal Antibody Production Analysis

An Insulin AutoAntibody (IAA) enzyme immunoassay kit, manufactured by Mercodia AB (Uppsala, Sweden) was purchased from ALPCO (Windham, NH), and used for antibody production analysis. Intended for use with human blood or plasma samples to screen for diabetes, this assay provides only qualitative measures of monoclonal antibody concentrations. This allowed relative antibody-level comparisons between control and experimental media samples taken at the same time, as well as concentration trend analysis for both systems.

The kit provided solutions and materials for one 96-well plate to be analyzed. The assay protocol as provided with the kit was followed, with one major exception.

Instead of a single 1:25 dilution in duplicate for each sample, three dilutions (1:1, 1:100 and 1:10,000) were performed in duplicate for each sample, for a total of 6 wells used for each sample. 12 media samples (6 matched pairs of experimental and control samples), and fresh S-MEM as used in the culture flasks, were each prepared. Therefore 78 of the 96 wells were used for sample measurements. Duplicates of a 6-point standard curve occupied another 12 wells, and 6 wells were unused.

Chapter 4 – Results and Discussion

Soluble Glutamine Synthetase Activity in Buffer

Reactant Concentration Effects

The five-factor, two-level glutamine synthetase activity experiment in DMEM buffer yielded 20 sets (10 treatments, each run in duplicate) of ammonia concentration profiles over time, which are presented in figures 4.1 through 4.10. Each profile was comprised of six ammonia concentration measurements, ending at a time beyond the apparent enzymatic reaction endpoint. For each of the 10 treatments, the two replicate profiles were combined into one composite profile. Since the initial ammonia concentrations of each pair of replicates differed somewhat due to experimental error (likely incurred mainly during solution preparation), the replicate profiles for each time point were normalized to eliminate these variations. This normalization was performed by multiplying each data point of the first replicate by a factor calculated as:

(Replicate 2 Initial Concentration [mM]) / (Replicate 1 Initial Concentration [mM]).

Curves were then fit to the resulting 10 composite treatment profiles. Initial attempts to fit curves of the form $y = Ax + Bx^2 + Cx^3 + D$, where y is ammonia concentration in mM and x is elapsed time in hours, yielded apparently good fits with high R^2 values (ranging from 0.95 to 0.98), but introduced curve-fitting artifacts. Based on the shape of the profiles, curves of the form $y = Ax + Bx^{1/2} + Cx^{1/3} + D$ and $y = Ax + Bx^{1/2} + C$ were next analyzed. Both forms yielded excellent fits, with average R^2 values of 0.98 and 0.97, respectively. However, like the third-order form first attempted, the

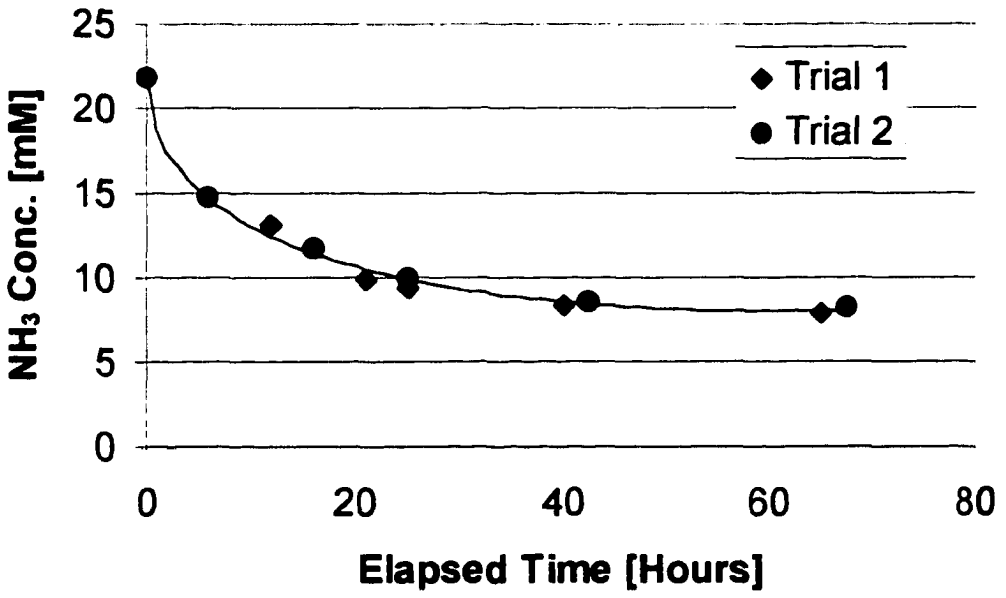

Figure 4.1 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 1: 20 mM Glu, 20 mM ATP, 30 mM Mg^{2+}, 0 mM Gln.

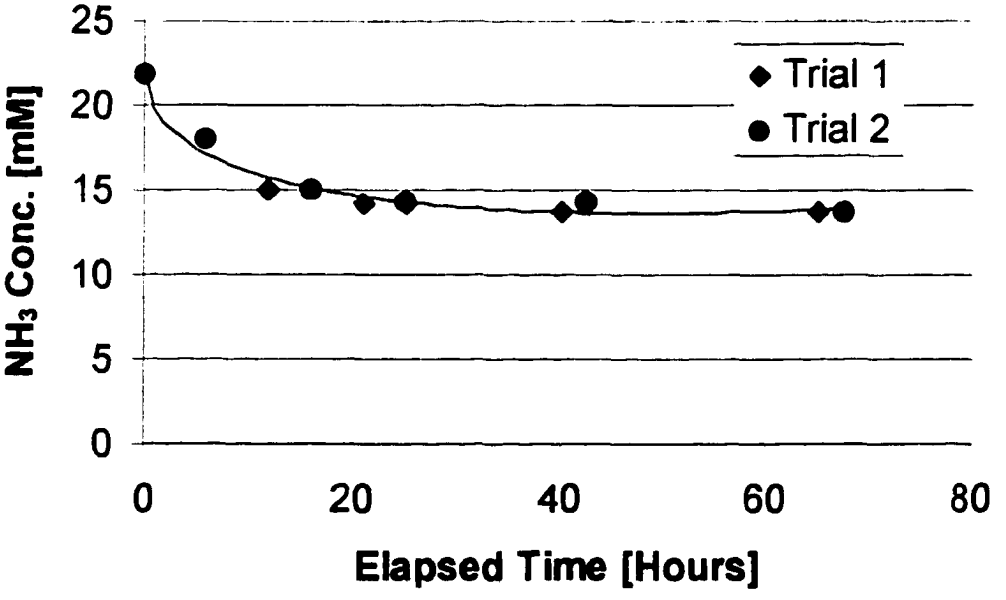

Figure 4.2 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 2: 10 mM Glu, 10 mM ATP, 30 mM Mg^{2+}, 0 mM Gln.

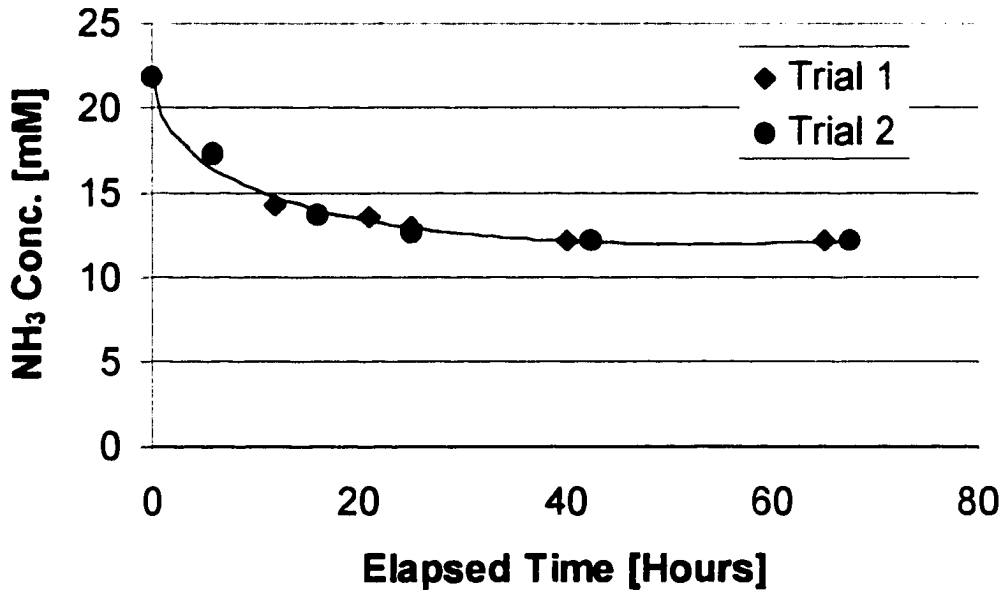

Figure 4.3 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 3: 20 mM Glu, 10 mM ATP, 15 mM Mg^{2+}, 0 mM Gln.

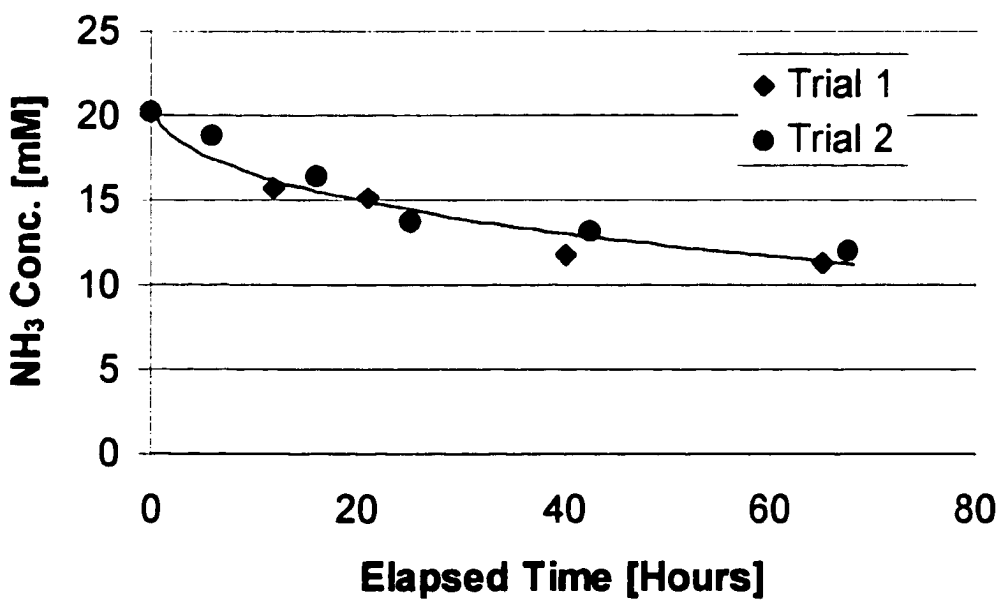

Figure 4.4 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 4: 10 mM Glu, 20 mM ATP, 15 mM Mg^{2+}, 0 mM Gln.

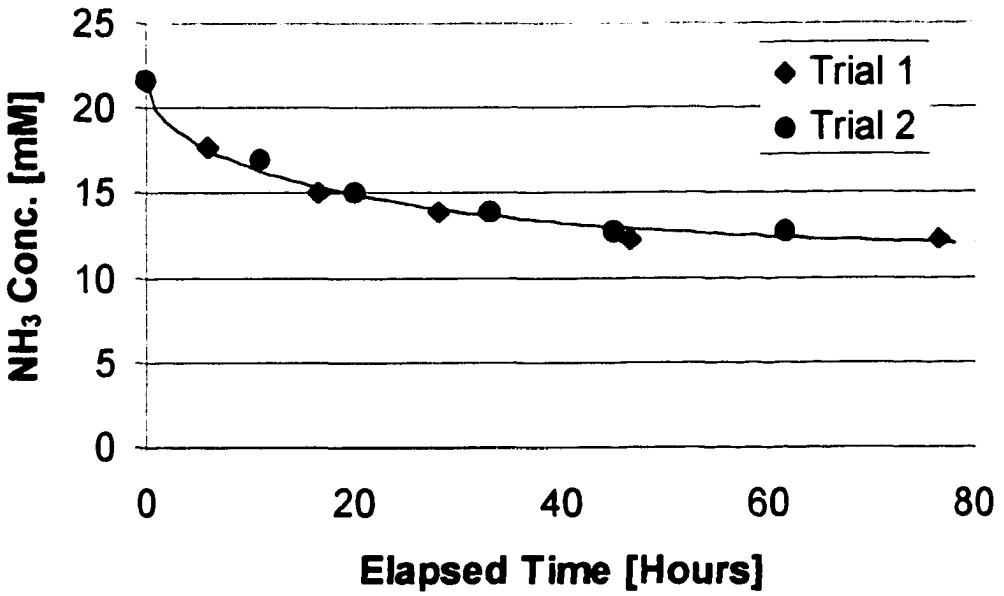

Figure 4.5 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 5: 10 mM Glu, 20 mM ATP, 15 mM Mg^{2+}, 5 mM Gln.

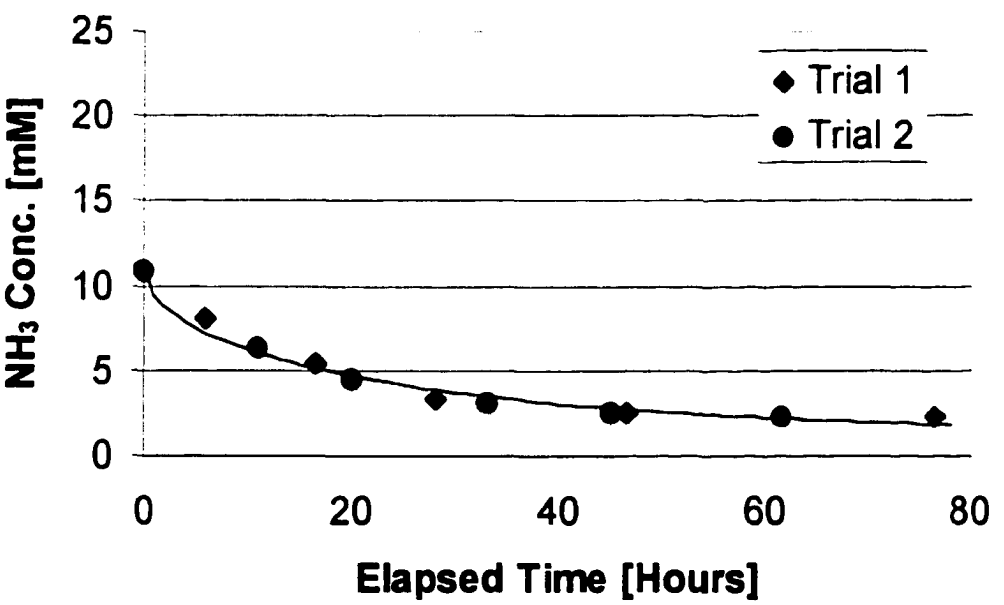

Figure 4.6 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 6: 10 mM Glu, 20 mM ATP, 15 mM Mg^{2+}, 0 mM Gln.

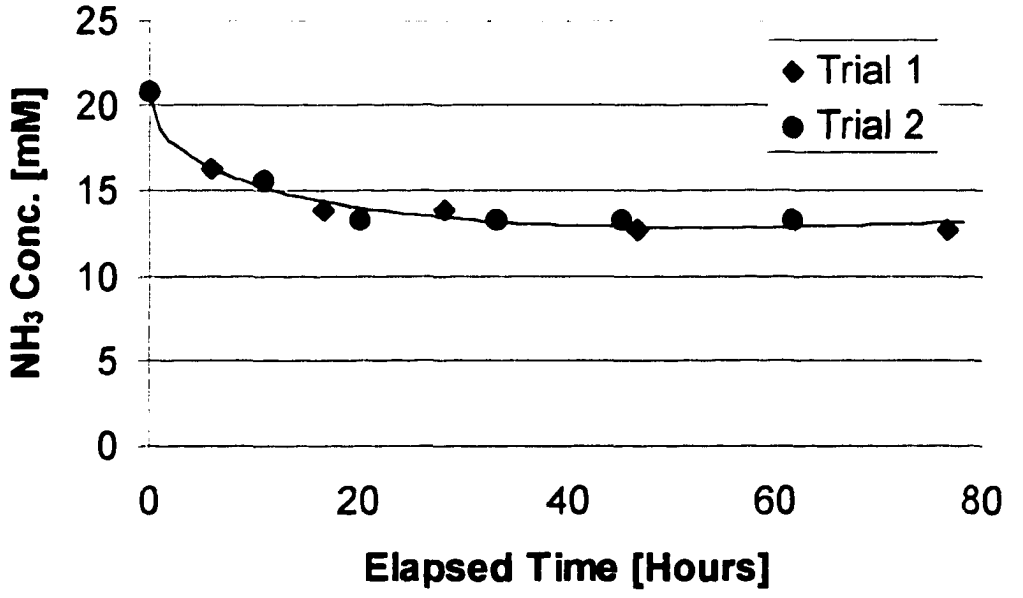

Figure 4.7 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 7: 10 mM Glu, 10 mM ATP, 15 mM Mg^{2+}, 0 mM Gln.

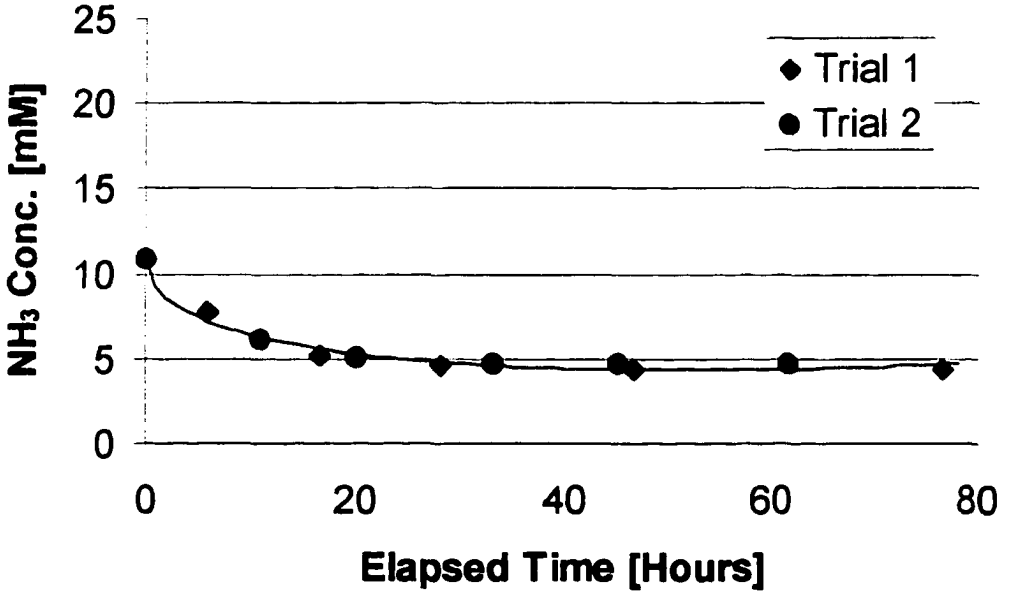

Figure 4.8 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 8: 10 mM Glu, 10 mM ATP, 15 mM Mg^{2+}, 5 mM Gln.

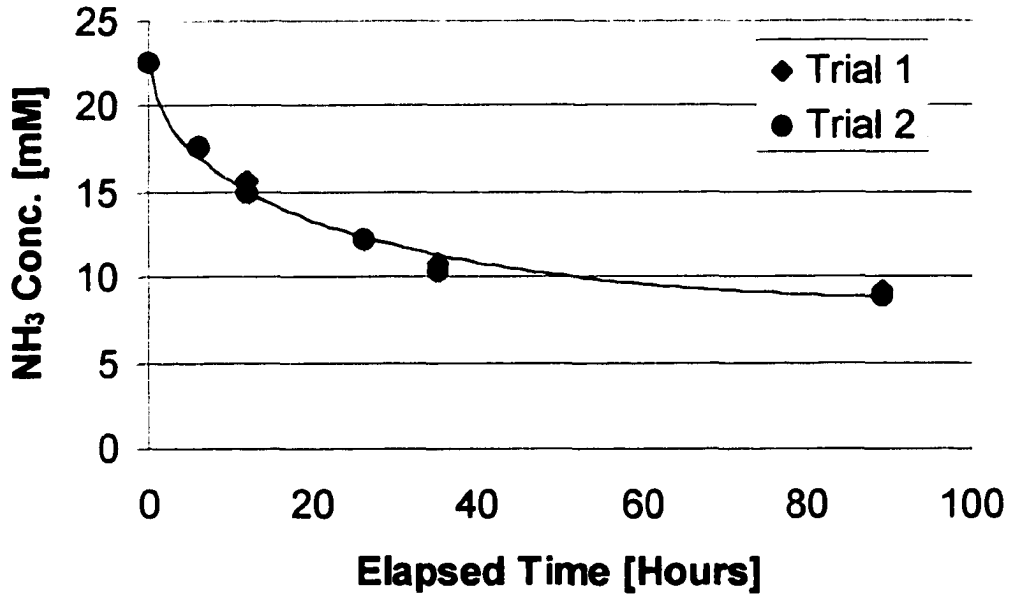

Figure 4.9 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 9: 20 mM Glu, 20 mM ATP, 15 mM Mg^{2+}, 0 mM Gln.

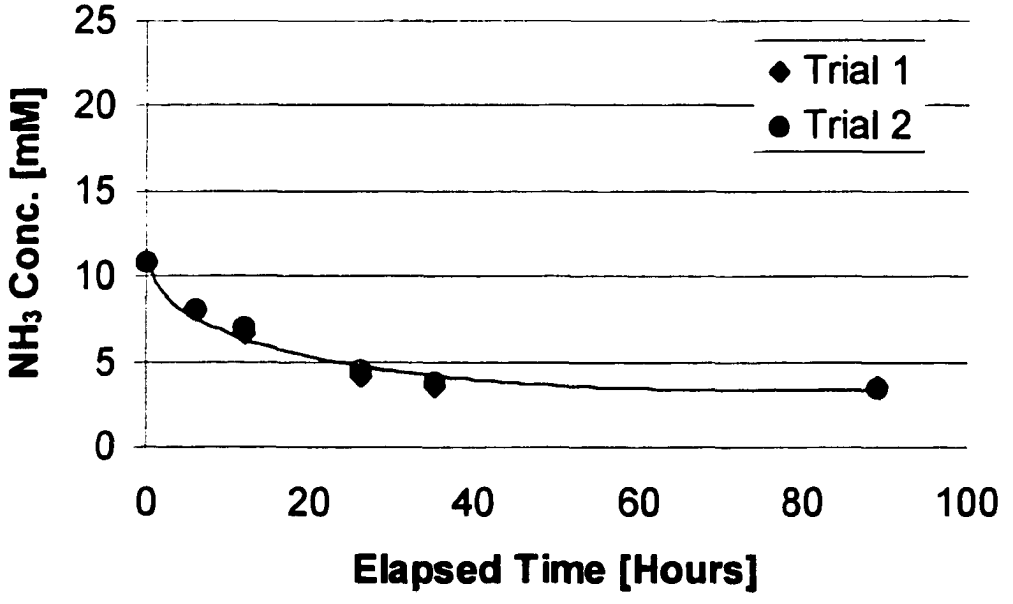

Figure 4.10 Soluble enzyme experiment: ammonia concentration vs. time. Treatment 10: 10 mM Glu, 20 mM ATP, 15 mM Mg^{2+}, 5 mM Gln.

former also introduced artifacts. The latter form $y = Ax + Bx^{1/2} + C$ did not yield artifacts and so were chosen. More information on the curve-fitting process is presented in Appendix 4. Interpolated values from these curves were used for treatment comparisons. Figures 4.1 through 4.10 show both replicate data sets for each treatment, as well as the fitted curve, over each treatment's entire duration. Interpolated values were required for the ensuing analysis as measurements were taken at differing times.

Using interpolated values from these fitted curves, two metrics were quantified for each experimental treatment: the initial 17-hour ammonia conversion rate in μmoles / unit of enzyme–hour, and the total 60-hour ammonia conversion in mM. These metrics were chosen to provide quantitative information on the effect of each of the five substrates of interest on reaction kinetics and completion, respectively. For the first metric, the initial 17-hour time period was chosen for two reasons. First, in the sepharose bead-immobilization thermostability study, to be described later in this chapter, samples were taken at 17 hours, and quantifying soluble enzyme conversion rates at this time point allowed relevant comparison of activities between soluble and immobilized glutamine synthetase. Second, for most of the 10 treatments, the fitted curves exhibited smaller deviations from the actual data points at this time than at most earlier times. For the second metric, the 60-hour point was used as for all treatments enzymatic ammonia conversion was at or very near completion by this time.

The individual effects of each of the five substrates on 17-hour initial ammonia conversion rate are presented in Table 4.1 and Figure 4.11, and the effects on 60-hour total ammonia conversion are presented in Table 4.2 and Figure 4.12. Since only 10 of

the $2^5 = 32$ total treatments necessary for a complete two-level, five-factor analysis were performed, only the main (individual) substrate effects could be quantified; interactive effects could not be evaluated. Tables 4.1 and 4.2 therefore present each comparison used in calculating the mean main effect of each substrate for several levels of the other components. For example, in the glutamine analysis, the "NAG – NA" comparison reveals the difference in conversion rate and total ammonia converted for the condition of high ammonia, ATP and glutamine ("NAG") compared to that observed with high ammonia and ATP only ("NA"). The second comparison for glutamine, "AG – A", represents the differences observed in these two metrics between the condition of high ATP and glutamine ("AG") and high ATP only ("A"). Large differences between these individual comparisons for each substrate suggest substantial substrate interactive effects. Given the number of conditions deemed feasible to evaluate experimentally, a complete analysis-of-variance, and therefore F-tests for effect significance were not feasible. Effect significance was therefore evaluated by comparing each substrate's mean main effect to the appropriate average value for the 10 experimental treatments. For the kinetics analysis, each substrate's mean main effect was compared to the mean 17-hour ammonia conversion rate for the 10 treatments, and in the reaction completion analysis, the main effects were compared to the mean treatment 60-hour ammonia conversion.

The kinetics results in Table 4.1 indicate that on the average an increased concentration of glutamate from 10 to 20 mM yields a positive effect on initial ammonia conversion rate of 0.28 μmoles/unit-hour. This is by far the largest effector of conversion rate, followed by Mg^{2+} at 0.13 μmoles/unit-hour. These two effects are likely significant,

Substrate	Comp. 1*	Effect 1**	Comp. 2*	Effect 2**	Comp. 3*	Effect 3**	Mean Effect**	% of Mean Treatment Rate
Glutamine	NAG-NA	0.15	AG-A	-0.06	–	–	0.05	7%
ATP	NA-N	-0.16	AG-G	-0.01	NUA-NU	0.07	-0.03	-5%
Mg^{2+}	NUAM-NUA	0.21	NM-N	0.05	–	–	0.13	18%
Glutamate	NU-N	0.16	NUA-NA	0.39	–	–	0.28	39%
NH_3	NA-A	-0.09	NAG-AG	0.12	–	–	0.01	2%

*Treatment Codes: N=High NH_3, A=High ATP, G=High glutamine, U=High glutamate, M=High Mg^{2+}

** In μmoles NH_3 converted/unit-hr

Table 4.1 Soluble enzyme experiment: substrate main effects on 17-hr. NH_3 conversion rate. Effects expressed in μmoles NH_3 converted/unit enzyme-hr.

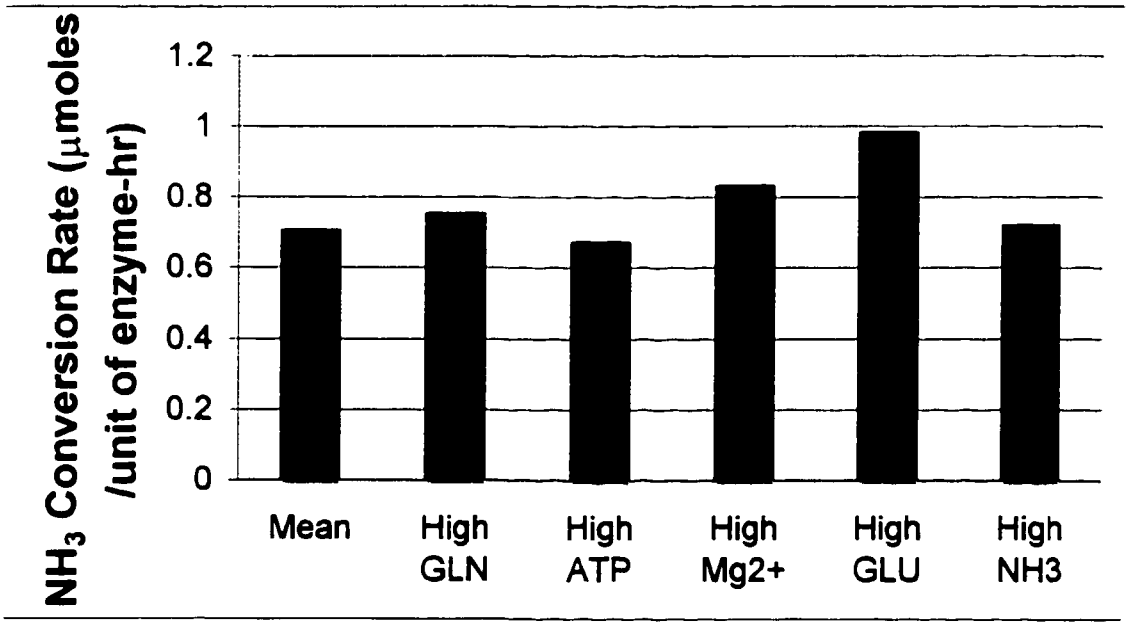

Figure 4.11 Soluble enzyme experiment: substrate main effects on 17-hr. NH_3 conversion rate.

Substrate	Comp. 1*	Effect 1**	Comp. 2*	Effect 2**	Comp. 3*	Effect 3**	Mean Effect**	% of Mean Treatment Conversion
Glutamine	NAG-NA	0.61	AG-A	-1.18	–	–	-0.28	-3%
ATP	NA-N	0.72	AG-G	0.99	NUA-NU	3.11	1.61	17%
Mg^{2+}	NUAM-NUA	0.94	NM-N	0.27	–	–	0.61	6%
Glutamate	NU-N	1.97	NUA-NA	4.36	–	–	3.16	34%
NH_3	NA-A	0.02	NAG-AG	1.81	–	–	0.91	10%

*Treatment Codes: N=High NH_3, A=High ATP, G=High glutamine, U=High glutamate, M=High Mg^{2+}

** NH_3 converted in mM

Table 4.2 Soluble enzyme experiment: substrate main effects on 60-hr. NH_3 conversion. Effects expressed in mM.

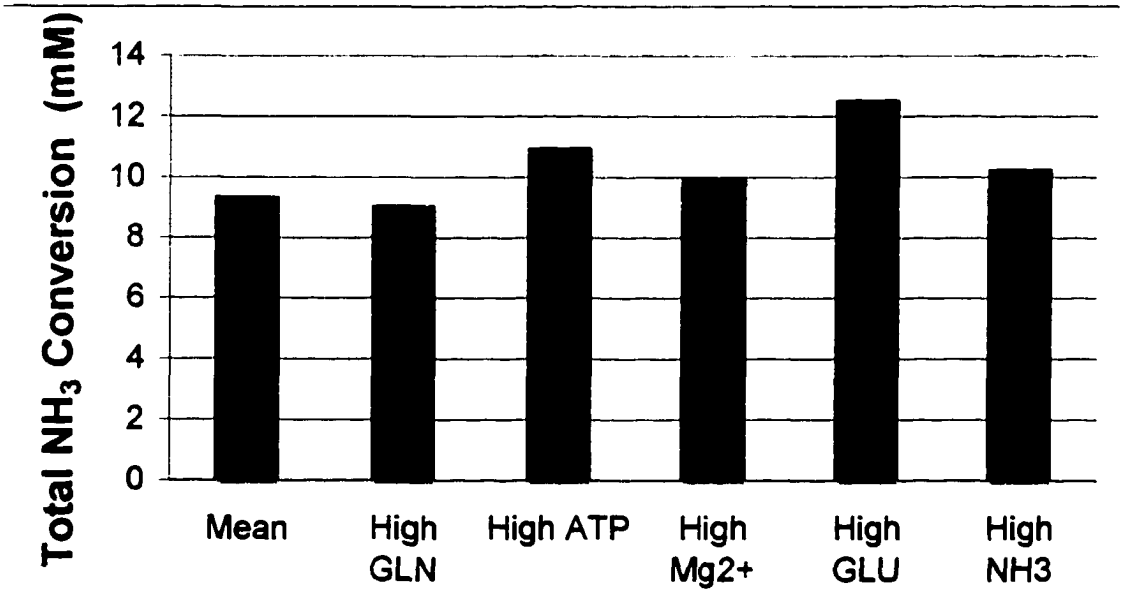

Figure 4.12 Soluble enzyme experiment: substrate main effects on 60-hr. NH_3 conversion.

representing 39% and 18% of the average treatment conversion rate (0.70 μmoles/unit-hour), respectively. In contrast, the effects of glutamine, ATP and ammonia are likely not significant, representing only 7%, -5% and 2% of the average treatment rate, respectively. These deviations are likely within the level of experimental error.

In the reaction completion analysis in Table 4.2, glutamate is once again the largest effector; 20 mM glutamate results in an average increase of 3.16 mM ammonia conversion than 10 mM glutamate. However, here ATP is the next largest effector, followed by ammonia, Mg^{2+}, and a negative effect from glutamine. This negative effect of glutamine is somewhat expected due to potential feedback inhibition effects. At 34%, 17% and 10% of the average treatment total ammonia conversion rate respectively, the effects of glutamate, ATP and NH_3 are likely significant; the other effects may not be.

Differences between comparisons for each substrate main effect calculation are quite large, which likely indicates the presence of interactive effects. Most of these differences between comparisons are somewhat intuitive. The positive effect of ATP is strong when there is an adequate supply of the other reactants. For example, the positive effect of ATP on total ammonia conversion of 3.11 mM calculated by "NUA – NU" (high ammonia, glutamate and ATP minus high ammonia and glutamate only) is much larger than the effect calculated by "NA – N" (high ammonia and ATP minus high ammonia only). In the latter comparison, the effect of limiting glutamate likely masks the effect of ATP, resulting in a relatively small effect. In contrast, the former comparison contains abundant glutamate, and the effect of limiting ATP dominates, yielding a much larger individual effect.

ATP Hydrolysis

For each of eight ATP/Mg^{2+} titrations performed as described in the previous chapter, the results of H^+ released upon addition of ATP was monitored. These are presented in Figure 4.13. The results presented suggest that the ratio of Mg^{2+} to ATP influences the proportion of ATP that degrades into ADP, P_i and H^+. The two treatments with the highest [Mg^{2+}]:[ATP] ratios, the 20 mM ATP, 30 mM Mg^{2+} and 10 mM ATP, 15 mM Mg^{2+} solutions, released the greatest amount of H^+ per volume of solution added. The four solutions with [Mg^{2+}]:[ATP] ratios of 1:1 were next, followed by the 20 mM ATP, 15 mM Mg^{2+} solution, and finally the 20 mM ATP, 0 mM Mg^{2+} solution, which released by far the least H^+ per volume added. These behaviors indicate that Mg^{2+} concentration, and particularly [Mg^{2+}]:[ATP] ratio, significantly influence the extent of spontaneous ATP hydrolysis in solution. This effect is further represented in Figure 4.14. In this figure, the estimated spontaneous ATP hydrolysis percentages are shown for the four 20 mM ATP solutions only. These four solutions have Mg^{2+} concentrations of 0 mM, 15 mM, 20 mM and 30 mM, and [Mg^{2+}]/[ATP] values of 0, 0.75, 1 and 1.5, respectively. As the figure shows, ATP hydrolysis scales linearly with [Mg^{2+}]/[ATP] over this range, and an increase in [Mg^{2+}]/[ATP] value from 0 to 1 results in an increase in ATP hydrolysis from 30% to 36% of ATP added.

These results were used to estimate the extent of ATP hydrolysis at pH 7.4, at which all glutamine synthetase experiments were maintained, by calculating apparent hydrolysis equilibrium constants and adjusting [H^+] to yield new [ADP]/[ATP] values. The values produced from this exercise yielded very large K_{eq}'s for the hydrolysis

reaction, indicating almost complete hydrolysis upon dissolution and adjustment to pH 7.4. These results were highly unrealistic when compared to observations from soluble enzyme experiments described earlier, perhaps due to the different buffering capacities of water and DMEM buffer. Reaction endpoints observed in ATP-limiting treatments of the soluble enzyme experiment described above seem to indicate hydrolysis of approximately 35% of the initial ATP over the experiments' duration.

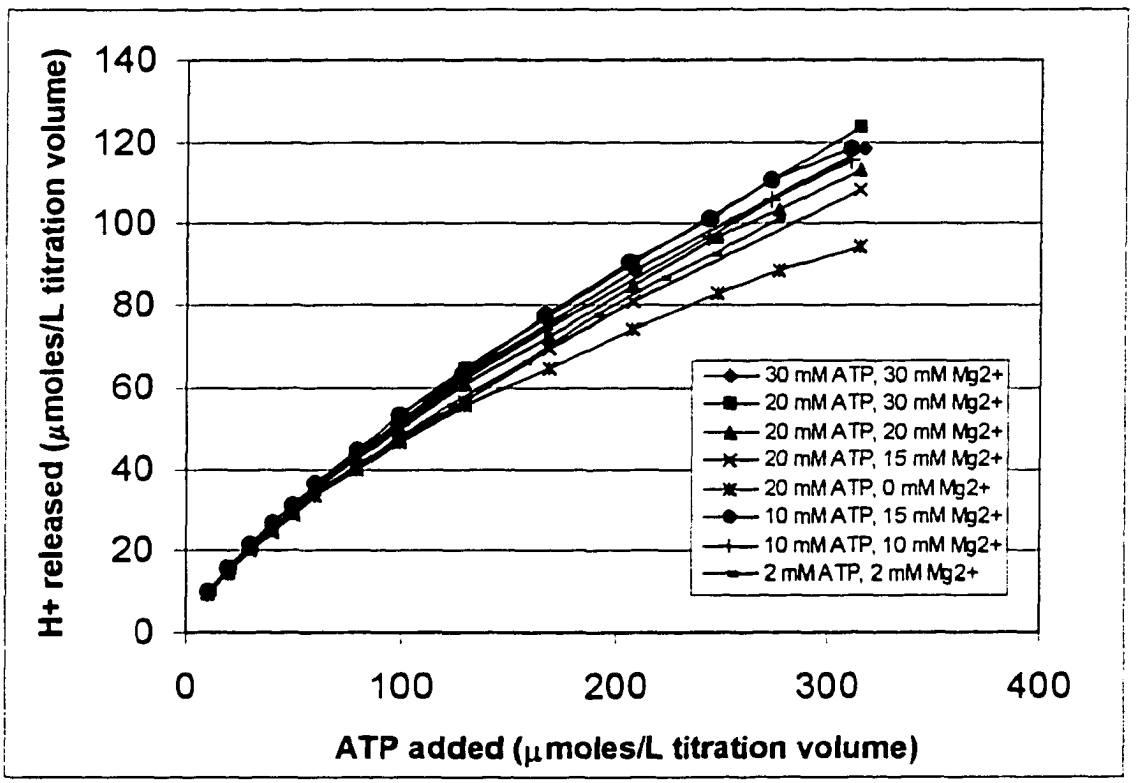

Figure 4.13 ATP hydrolysis experiment: H^+ released per ATP added.

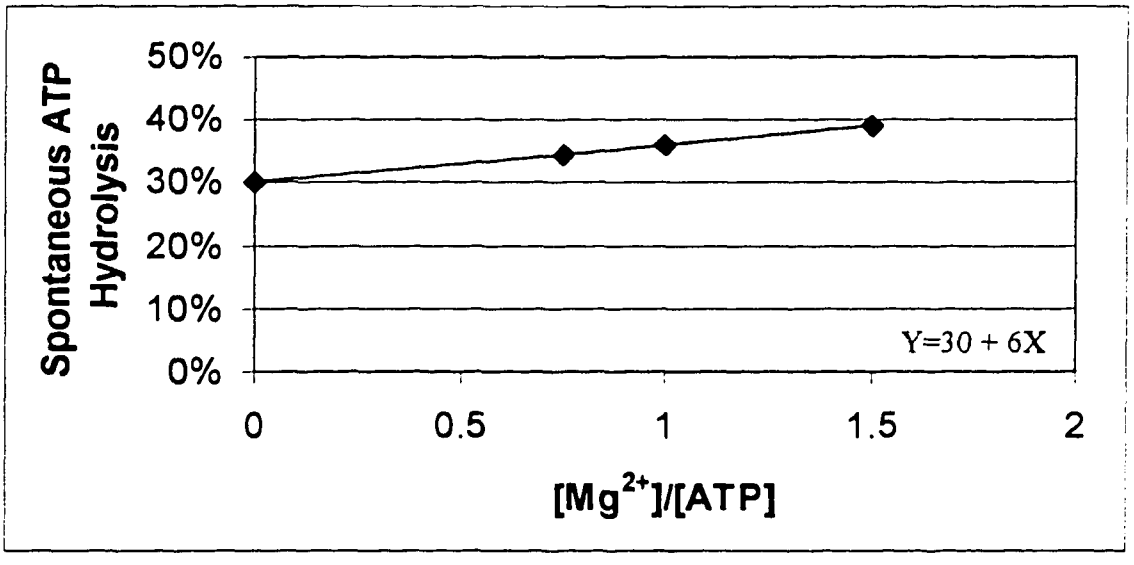

Figure 4.14 ATP hydrolysis experiment: spontaneous ATP hydrolysis vs. Mg^{2+}:Initial ATP ratio, from titrations with 20 mM ATP solutions.

Enzymatic Reaction Equilibrium Analysis

To provide information about the anticipated endpoint of glutamine synthetase-catalyzed ammonia conversion, equilibrium constants for the reaction

$$\text{Glutamate} + NH_3 \rightarrow \text{Glutamine}$$

$$K_{eq} = [GLN]/[GLU][NH_3]$$

were sought. This information was desired for two main reasons: 1) to determine if there is a driving force for the backward reaction, and 2) to allow the estimation of reaction completion in subsequent enzyme applications. Four experimental treatments were selected for this analysis: two from the five-factor, two-level experiment described above (treatments 6 and 10), and two from another soluble enzyme experiment performed at very low reactant concentrations. These four were chosen because they were run with abundant ATP; treatments with exhausted ATP were avoided due to the premature

endpoints observed under these conditions. This premature end of the reaction implies that an equilibrium had not been reached.

Table 4.3 displays the results of this analysis. Initial concentrations were known, and final concentrations for each of the three substrates listed (ammonia, glutamate and glutamine) were calculated based on initial concentrations and the amount of ammonia converted. The equilibrium constants calculated ranged from 1.40 to 2.86, with an average of 2.13, which represents only a small driving force for the reverse reaction. For example, a solution with starting concentrations of 10 mM ammonia, 10 mM glutamate, 0 mM glutamine and non-limiting ATP would be expected to reach a reaction endpoint at ammonia, glutamate and glutamine concentrations of approximately 2 mM, 2 mM and 8 mM, respectively. This represents approximately 80% ammonia conversion. It should be noted that this ammonia conversion completion estimate is dependent on the initial reactant concentrations assumed here; other initial concentrations would yield different reaction completion estimates.

	Initial Concentrations (mM)			Final Concentrations (mM)			
Treatment	NH_3	Glutamate	Glutamine	NH_3	Glutamate	Glutamine	K_{eq}
1	10.8	10	0	2.2	1.4	8.6	2.79
2	10.8	10	5	3.4	2.6	12.4	1.40
3	1.8	2	4	1.6	1.8	4.2	1.46
4	1.4	1	4	1.4	1	4	2.86

Table 4.3 Enzymatic reaction equilibrium analysis.

Soluble Glutamine Synthetase Activity in Culture Media

Activity in Buffer, Buffer Plus Ca^{2+} and DMEM

DMEM contains 1.8 mM Ca^{2+}, a substrate known to be highly inhibitory to glutamine synthetase activity (Kingdon and Stadtman, 1967). To quantify this effect, and to evaluate DMEM's suitability for further use in this study, an experiment was performed comparing soluble glutamine synthetase activity in DMEM buffer, in DMEM buffer with 1.8 mM added Ca^{2+}, and in DMEM itself. To each solution was added 10 units of glutamine synthetase and identical reactant concentrations: 10 mM NH_3, 10 mM glutamate, 20 mM ATP and 15 mM Mg^{2+}. In addition to initial samples, three ammonia concentrations were measured at later times, covering a span of 30 hours. The three ammonia concentration profiles were normalized to a common initial concentration using the same normalization method described previously.

The results of this experiment are presented in Figure 4.15. As this graph clearly displays, the ammonia conversion rate in buffer only is much faster than in either buffer plus Ca^{2+} or DMEM. In addition, the conversion rates in buffer plus Ca^{2+} and DMEM are quite similar, representing 42% and 34% respectively of the buffer-only conversion rate over the entire 30-hour experiment duration.

These results strongly suggest that: 1) DMEM does not provide an environment conducive to high glutamine synthetase activity, and 2) this adverse characteristic is potentially due predominately to the Ca^{2+} present in the medium.

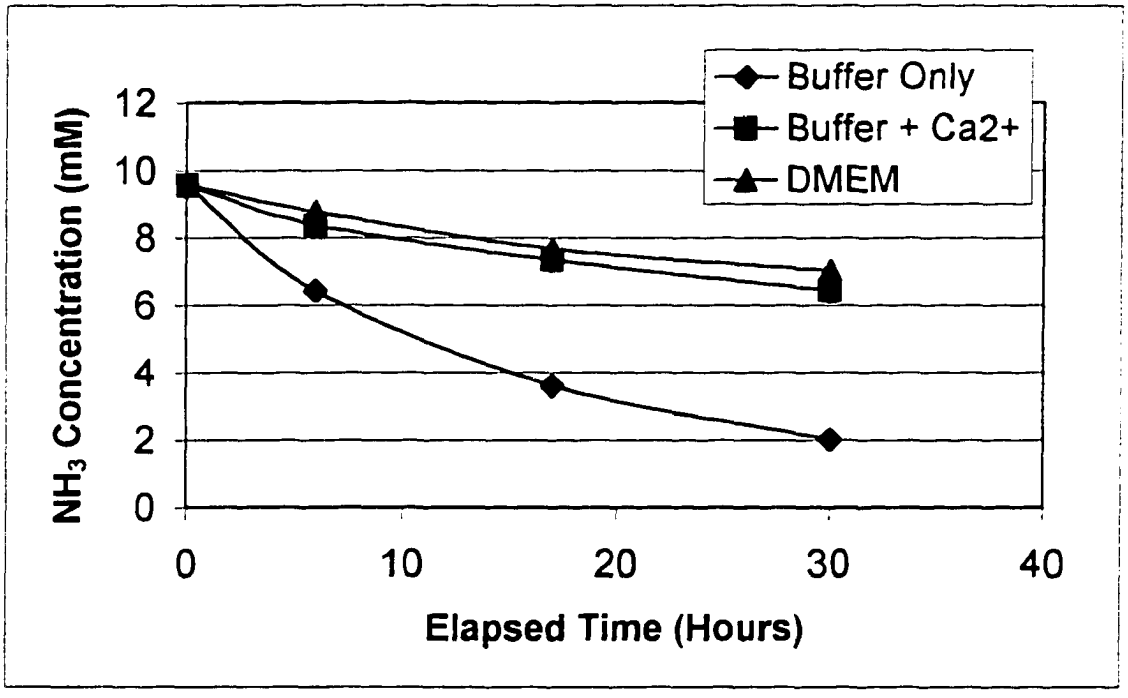

Figure 4.15 Soluble Enzyme Activity in Buffer, Buffer With 0.265 g/L Ca^{2+} and DMEM.

The minimal difference in activities between the buffer plus Ca^{2+} and DMEM treatments may be due to experimental or measurement error, but may also be the result of a slight feedback-inhibition effect from DMEM's 4 mM glutamine, or to other effects not quantified.

Activity in S-MEM and DMEM, With and Without Serum

Due to the poor glutamine synthetase activity observed in DMEM, S-MEM was evaluated as a substitute. Unlike DMEM, S-MEM contains no Ca^{2+}. In this experiment, soluble enzyme activity was compared in five bulk solutions: 1) DMEM buffer, 2)

DMEM without serum, 3) DMEM with 10% horse serum, 4) S-MEM without serum, and 5) S-MEM with 10% horse serum. To each solution was added 10 units of soluble glutamine synthetase as well as 5 mM each of Mg^{2+}, glutamate, ATP and ammonia. Samples were collected from each treatment after 12 hours, and the ammonia conversion rate in each quantified. These rates are displayed in Figure 4.16.

As the graph shows, the highest enzyme activity was observed in S-MEM without serum, followed closely by DMEM buffer and S-MEM with serum. As expected, the conversion rate in DMEM, both with and without serum, is much lower. In both media, serum had an inhibitory effect, reducing the conversion rate by 15% and 35% in S-MEM and DMEM, respectively.

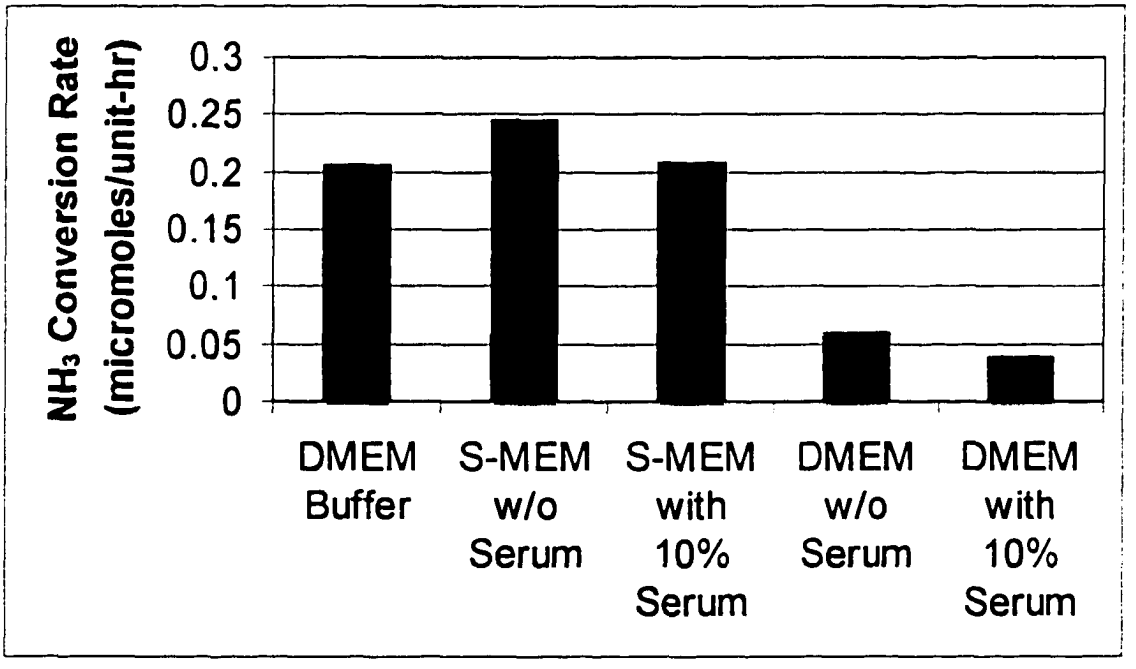

Figure 4.16 Soluble Enzyme Activity Analysis: 12-Hour NH_3 Conversion Rate Comparison in Various Bulk Solutions.

This effect is potentially attributable to Ca^{2+} in the serum. The reason for superior activity in S-MEM over DMEM buffer is not clear, but may be due to differences in buffer compositions beyond Ca^{2+} effects.

Immobilization of Glutamine Synthetase

Covalent Binding to CNBr-Activated Sepharose Beads

Coupling Protocol Comparisons

The two sepharose bead immobilization protocols described in the previous chapter are similar, but differ in their post-coupling rinsing procedures. In the Modified Sigma Protocol, the beads are rinsed with a pH 4 solution, whereas in the Modified Hermanson Protocol, a pH 7 buffer is used. To quantify the effect of this procedural difference, glutamine synthetase was immobilized using both protocols, and the resulting residual activities compared. A third treatment was also included in the experiment: glutamine synthetase immobilized via the Modified Hermanson Protocol, but with the blocking of unreacted groups omitted. 50 units of enzyme were immobilized according to each of the three protocols, and the resulting beads transferred to identical volumes of DMEM buffer containing 10 mM NH_3, 10 mM glutamate, 20 mM ATP and 15 mM Mg^{2+} (treatment 6 from the soluble enzyme five-factor experiment). Samples were taken from each treatment after 17 hours and ammonia conversion rates calculated. These rates were compared to the soluble enzyme conversion rate calculated for treatment 6 of the soluble enzyme five-factor experiment, and each protocol's residual activity calculated. These results are presented in Table 4.4.

Coupling Protocol Used	17-Hour NH_3 Conversion Rate (μmoles NH_3/unit-hr)	Residual Activity
Sigma Protocol	0.016	2.5%
Hermanson Protocol - Blocking	0.047	7.4%
Hermanson Protocol - No Blocking	0.054	8.5%

Table 4.4 CNBr-Activated Sepharose Bead Immobilization: Coupling Protocol Comparisons. Residual activity is defined as (Immobilized Enzyme Activity/Soluble Enzyme Activity) for the same number of units of enzyme.

As the table indicates, much greater residual activities were obtained from the two Hermanson Protocol variants than from the Sigma Protocol. This suggests that exposure to the pH 4 buffer used in the Sigma Protocol had an adverse, and possibly chaotropic effect on the immobilized enzyme. Of the two Hermanson Protocol variants, the "No-blocking" method yielded slightly higher activity. However, this benefit was not deemed significant enough to favor this variant in subsequent experiments, due to the potential for undesired binding of unblocked bead functional groups to other substrates or proteins in culture media. The Hermanson Protocol with blocking was therefore employed in future analyses.

Thermostability Analysis

50 units of glutamine synthetase were immobilized on CNBr-activated sepharose beads via the Hermanson Protocol with blocking. The resulting beads were transferred to

DMEM buffer with "Treatment 6" substrate concentrations, as in the previous experiment, and incubated at 37 °C. In addition to an initial sample, samples were taken at 6 and 17 hours, and their ammonia concentrations measured. After an elapsed time of between 2 and 5 days, the beads were rinsed thoroughly and placed in fresh "Treatment 6" solution, and the process repeated. 10 iterations were performed over 30 days, and the degradation of enzyme activity was evaluated using 17-hour ammonia conversion rates. These results are shown in Figure 4.17. Enzyme degradation shows a relatively linear trend, with a half-life of approximately 24 days.

Entrapment Within κ-Carrageenan Gel Beads

Evaluation of κ-carrageenan beads as a glutamine synthetase carrier was performed in two steps. First, beads were formed using several different carrageenan concentrations and two different hardening solutions, 0.3 M $MgCl_2$ and 0.3 M KCl, without the addition of any enzyme. This step was employed to identify the best concentration/hardening solution combination for use in actual enzyme immobilization. Second, glutamine synthetase was entrapped within carrageenan beads and the immobilized enzyme's activity observed in DMEM buffer and DMEM, both with "Treatment 6" reactant concentrations.

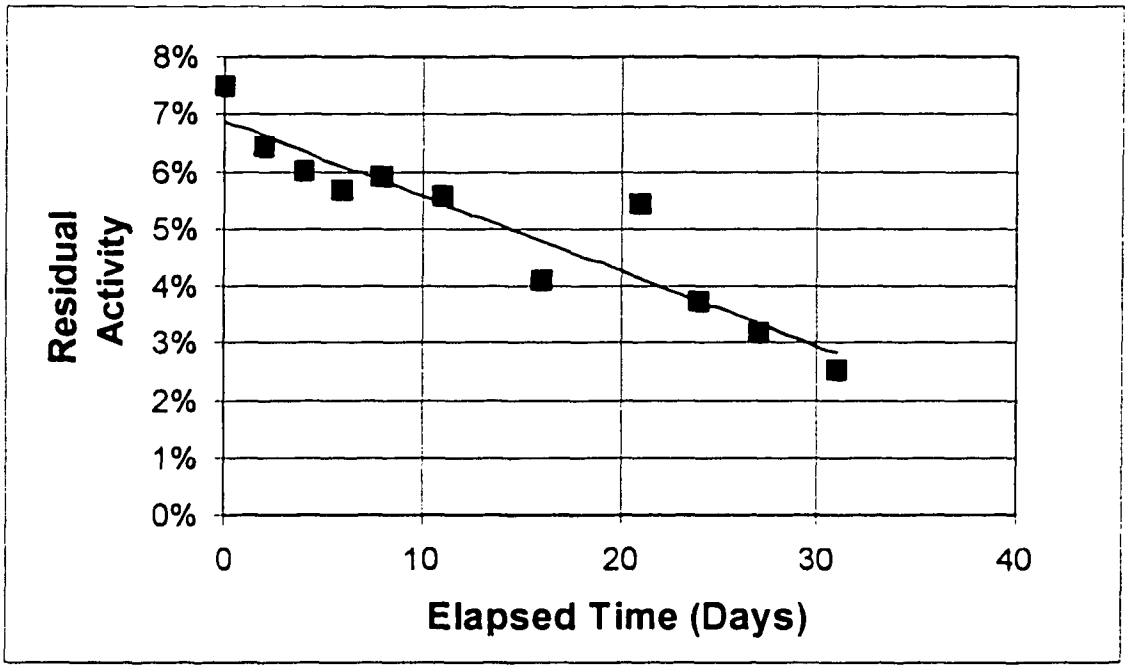

Figure 4.17 CNBr-Activated Sepharose Immobilization: Thermostability Analysis. Observed half-life is approximately 24 days.

The results from these studies are presented in Table 4.5. Beads hardened in 0.3 M $MgCl_2$ were highly unstable, and dissolved completely within 6 hours. Similarly, beads hardened with 0.3 M KCl also had very poor stability in water, likely due to the lack of cations in solution. Presumably, K^+ would diffuse out of the gel, thus weakening the mechanical stability. When maintained in DMEM buffer, KCl-hardened beads with no enzyme displayed excellent stability, showing no signs of degradation after 2 months. These characteristics were shared by beads composed of both 1.75% and 2.5% carrageenan. Unfortunately, glutamine synthetase entrapped within KCl-hardened beads yielded no measurable activity, and the beads displayed poor stability in both buffer and DMEM.

Hardening Solution	Carrageenan Conc. (% w/v)	Enzyme Added?	Bulk Solution	Bead Longevity @ 37 °C	Enzyme Activity
0.3 M $MgCl_2$	1.75	N	D.I. H_2O	Dissolved within 6 hours	N/A
0.3 M $MgCl_2$	2.5	N	DMEM Buffer	Dissolved within 6 hours	N/A
0.3 M KCl	1.75	N	D.I. H_2O	Dissolved within 6 hours	N/A
0.3 M KCl	1.75	N	DMEM Buffer	> 2 months	N/A
0.3 M KCl	2.5	N	D.I. H_2O	Dissolved within 6 hours	N/A
0.3 M KCl	2.5	N	DMEM Buffer	> 2 months	N/A
0.3 M KCl	2.5	Y	DMEM Buffer	Breaking up within 18 hours	0
0.3 M KCl	2.5	Y	DMEM	Breaking up within 6 hours	0

Table 4.5 κ-Carrageenan Immobilization Results.

Encapsulation Within Dialysis Tubing

20 units of glutamine synthetase were encapsulated in dialysis tubing, and transferred to 20 ml of S-MEM with 10% horse serum with "Treatment 6" reactant concentrations. Another treatment composed of 5.5 units of soluble glutamine synthetase in 5.5 ml of the same solution was also prepared. These two treatments were both incubated at 37 °C, and samples taken at 6 and 17 hours. To provide a thermostability comparison between soluble and encapsulated enzyme, another 5.5 units of soluble enzyme was placed in the same incubator, and maintained for 16 days at 37 °C. After 16 days, the dialysis tubing-encapsulated enzyme and the incubated soluble enzyme were

transferred to fresh bulk solutions, identical in composition and volume to those used above, and sampling at 6 and 17 hours was again performed. 17-hour ammonia conversion rates were calculated for both soluble and encapsulated enzyme from both runs, and the results presented in Figure 4.18.

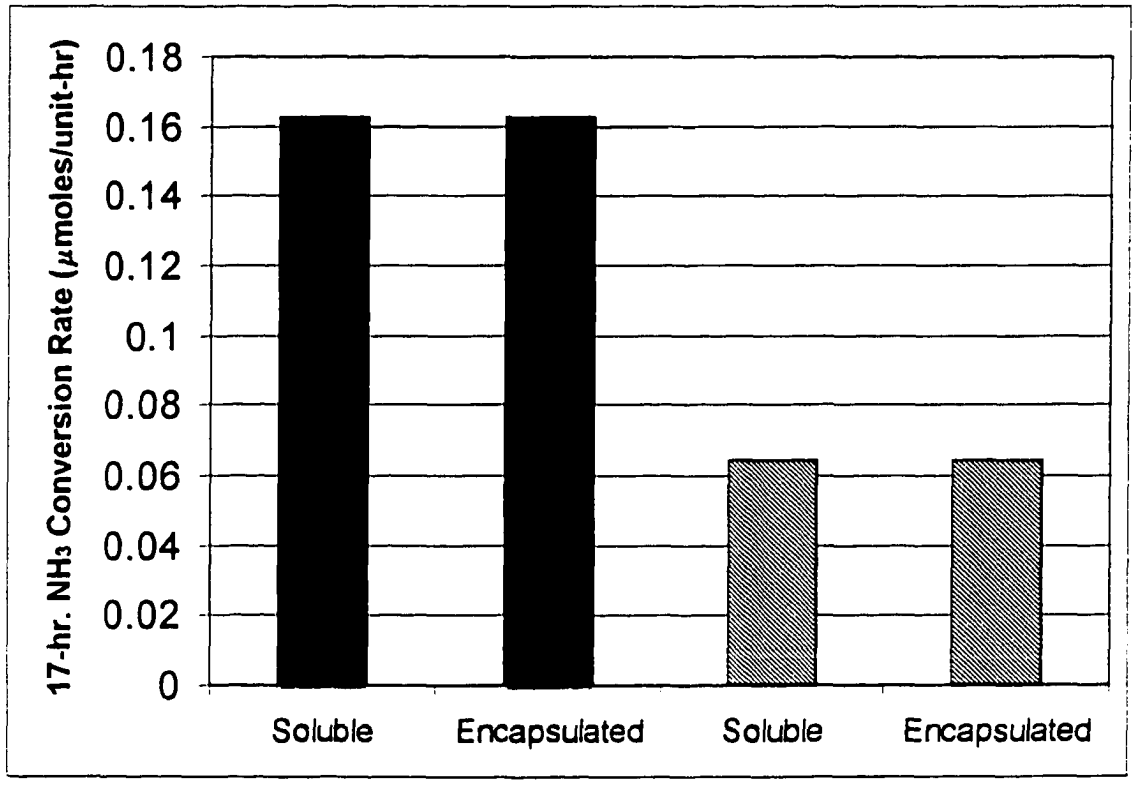

Figure 4.18 Soluble vs. encapsulated enzyme ammonia conversion rate comparisons. The striped bars represent rates obtained from the second run, performed 16 days after the first.

In both the first and second runs of the experiment, soluble and encapsulated enzyme exhibited identical conversion rates. The degradation in enzyme activity after 16 days for both soluble and encapsulated was also identical, dropping 60 % during this period. Compared to sepharose bead-immobilization, dialysis tubing encapsulation

yields far superior residual activity (100% compared to approximately 8%). However, if the activity degradation exhibited by encapsulated and soluble enzyme is assumed to follow a linear trend, both of their apparent half-lives are approximately 13 days. Under these assumptions, covalent binding of glutamine synthetase to sepharose beads provides approximately 45% improvement in thermostability over soluble enzyme at 37 °C.

Hybridoma Sensitivity To Enzyme Reactants

Cell density profiles for the eight treatments of the four-factor, two-level cell sensitivity experiment are presented in Figures 4.19 and 4.20. The four treatments represented in Figure 4.19 display classic cell life cycles, with clear lag, log, stationary and death phases. Of these four, treatment 1, the control treatment (with no added enzyme reactants), displayed the best cell-growth characteristics, both in terms of maximum cell density and maximum log-phase growth rate. Treatment 4 (high ammonia and glutamate) was next, reaching approximately 75% of the control maximum cell density, and treatments 3 (high ammonia and Mg^{2+}) and 2 (high Mg^{2+} and glutamate), each attained a maximum cell density of approximately 50% of the control.

Treatments 5-8 (Figure 4.20), the four treatments with high ATP, exhibited very different behavior. Of these four, the maximum cell density attained was approximately 200,000 cells/ml (by treatment 5, with high Mg^{2+} and ATP), which is approximately 1/6 of the control treatment maximum. The two treatments of this group that did not contain Mg^{2+}, treatments 6 and 7, exhibited no apparent cell growth, and were both reduced to essentially zero viability after only 25 hours. In contrast, the two treatments that did

contain Mg^{2+}, although far from optimal, each yielded cell density increases of approximately 100% over initial densities before the onset of the death phase. These results suggest that: 1) ATP exhibits a substantial cytotoxic effect, and 2) Mg^{2+} has a significant buffering effect on this ATP toxicity on hybridomas.

Based on the results from these eight treatments, the main effects of each of the four reactants on maximum cell density were calculated, and are displayed in Table 4.6. As the previous figures suggest, ATP had by far the largest negative effect. Mg^{2+}, glutamate and NH_3 all had similar but much smaller adverse effects.

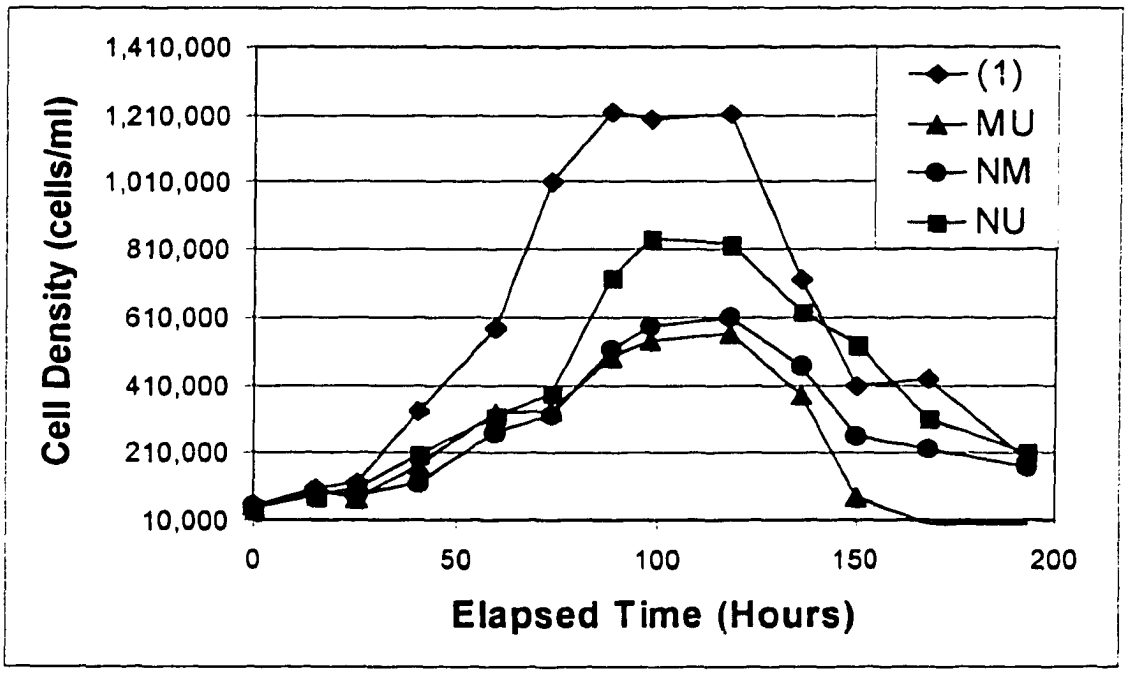

Figure 4.19 Hybridoma sensitivity experiment: cell density profiles, treatments 1 – 4. (1) = control, M = high Mg^{2+}, U = high glutamate, N = high NH3.

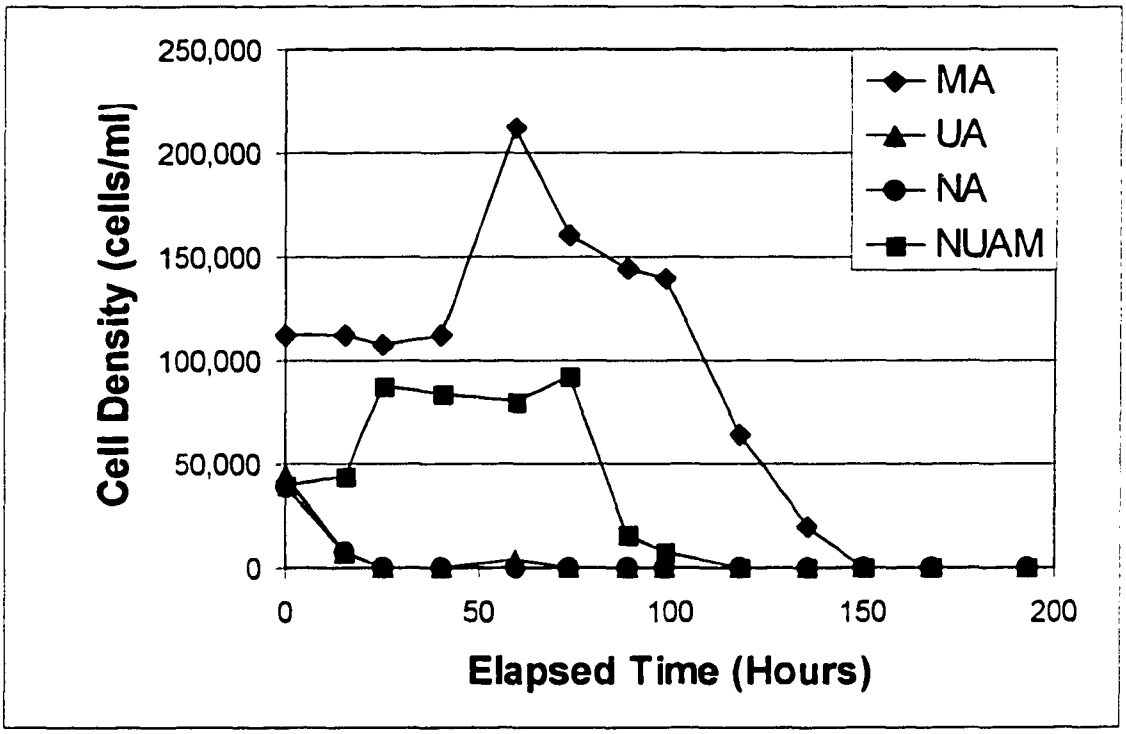

Figure 4.20 Hybridoma sensitivity experiment: cell density profiles, treatments 5-8. M = high Mg^{2+}, U = high glutamate, N = high NH_3, A = high ATP.

Substrate	Effect on Maximum Cell Density (cells/ml)
3 mM NH_3	-113,000
10 mM Glutamate	-135,000
10 mM ATP	-711,000
8 mM Mg^{2+}	-169,000

Table 4.6 Hybridoma sensitivity experiment: substrate main effects on maximum cell density in cells/ml.

Due to the unusual life cycles exhibited by treatments 5 through 8, standard time-based metrics, such as log-phase cell growth rate, did not provide meaningful results or treatment comparisons, and were therefore not included in the analysis.

Immobilized Glutamine Synthetase in an Active Hybridoma Cultivation

Analysis of Enzymatic Ammonia Conversion

The control and experimental culture flasks were maintained identically, with no additions or supplementation of any kind, until the 65-hour mark, when their ammonia concentrations reached 3 mM. The ammonia accumulation profiles for both systems up to this point are presented in Figure 4.21. As this figure shows, ammonia accumulation prior to the incorporation of enzyme-catalyzed ammonia conversion in the experimental system follow very similar patterns for both systems. After 65 hours, encapsulated glutamine synthetase was first introduced into the reaction chamber, and the first batch of 100 ml of experimental culture media subjected to enzymatic ammonia conversion. A total of 6 batches were transferred in the reaction chamber from this point to the termination of the experiment. The extent of ammonia conversion observed in each batch is detailed in Table 4.7.

The enzymatic conversion of ammonia proceeded in a sporadic fashion. The most substantial ammonia conversion, from 3.1 mM to 2.7 mM, was observed in the first batch, which was initiated at time 65 hours. In batch 2, no ammonia conversion was detected. At this time, a milky white residue was observed lining the inside surface of the Mg^{2+} solution reservoir; this was most likely Mg^{2+} precipitating out of solution. A new

batch of Mg^{2+} solution was therefore prepared and used in batch 3. Despite this, batch 3 also exhibited no enzymatic activity.

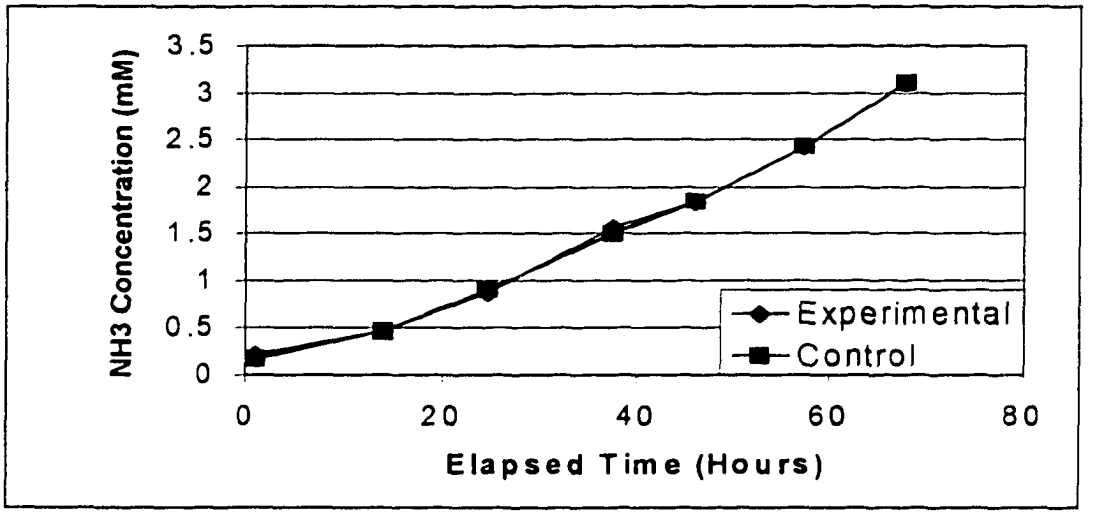

Figure 4.21 Final bioreactor experiment: ammonia accumulation prior to enzymatic ammonia conversion.

A potential cause for the lack of enzymatic activity lies in the resistance to transport through the dialysis tubing membrane. Some of the encapsulated hybridoma cells initially leaked from the agarose beads and proliferated. These accumulating cells were clearly visible in the culture medium, and could cause the observed lack of enzymatic activity by clogging the dialysis membrane. To address this problem, the soluble glutamine synthetase was transferred to new dialysis tubing prior to use in batch 4. This batch yielded 0.3 mM of ammonia conversion, suggesting that dialysis tubing fouling was indeed responsible for the previous poor results. The enzyme was therefore transferred to new dialysis tubing prior to batches 5 and 6 as well. Despite this action, batch 5 yielded no ammonia conversion. The Mg^{2+} solution reservoir was again observed

to contain a precipitate. Prior to batch 6, both the Mg^{2+} solution and the dialysis tubing were changed, and enzymatic activity was restored, with a 0.3 mM ammonia reduction in 11 hours. These observations suggest that both dialysis tubing fouling and precipitation of Mg^{2+} in its reservoir prior to use were responsible for the poor enzymatic ammonia conversion observed during this experiment.

Batch #	Elapsed Time (hours)	Rxn. Chamber Residence Time (hours)	Initial NH_3 (mM)	Final NH_3 (mM)	NH_3 Converted (mM)	Comment Code
1	65	9.5	3.1	2.7	0.4	
2	77	8.5	3.2	3.2	0.0	
3	85	10	3.5	3.5	0.0	Mg^{2+}
4	93	17	3.7	3.4	0.3	D.T.
5	115	14.5	4.1	4.1	0.0	D.T.
6	130	11	4.3	4.0	0.3	Mg^{2+}, D.T.

Comment Codes: Mg^{2+} -- Prepared fresh Mg^{2+} solution prior to current batch
D.T. – Re-encapsulated glutamine synthetase in new dialysis tubing prior to current batch

Table 4.7 Final Bioreactor Experiment: experimental spinner flask media ammonia conversion batch summary.

Ammonia Accumulation Analysis

Ammonia concentrations were measured for all experimental and control culture media samples, and are presented for the experiment's entire duration in Figure 4.22. As previously discussed, ammonia accumulated in an almost identical manner in the

experimental and control culture spinner flasks before the encapsulated glutamine synthetase was introduced into the experimental system after 65 hours. After this point, the ammonia concentration in the control system remained relatively constant, and the experimental ammonia concentration continued to increase.

The behavior exhibited by the control system is consistent with expectations: when glutamine was exhausted, cellular metabolism likely ceased. However, the continued ammonia accumulation in the experimental system was unexpected. One possible explanation of this behavior is that enzymatic ammonia conversion, although not substantial enough to maintain ammonia at a constant low concentration, did provide enough glutamine to maintain cellular metabolism. The hybridomas may have continued to metabolize other amino acids present in the culture medium or the animal serum.

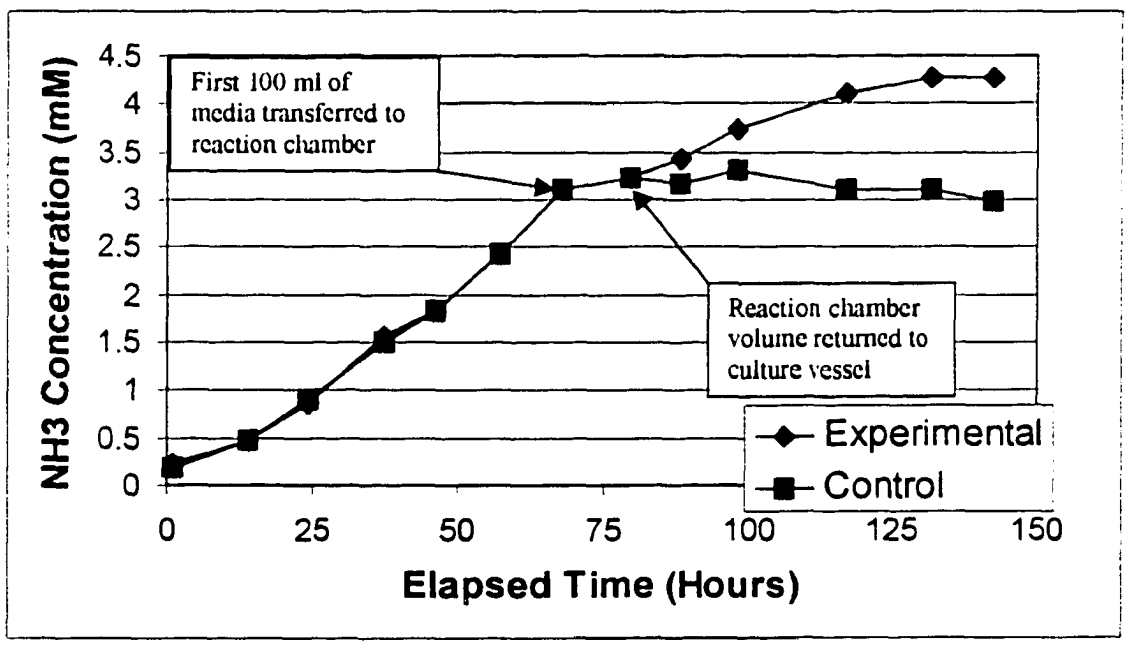

Figure 4.22 Final bioreactor experiment: NH_3 accumulation over time.

Suspension Cell Density Analysis

For this experiment, hybridomas were immobilized in agarose with the objective of minimizing the clogging of the dialysis tubing in the reaction chamber. However, as previously mentioned, cell leakage during the immobilization process resulted in substantial suspension cell populations in both the experimental and control systems. Since agarose-immobilized cell densities are difficult to accurately quantify, suspension cell densities were measured, and used as an indicator of overall culture health.

Viable suspension cell density profiles for both the experimental and control systems are presented in Figure 4.23. Differences between the two profiles are slight, and, for most data points, within the experimental error expected from hemocytometer cell counts (often as much as 15%). This similarity seems to suggest that either the added reactants (ATP and glutamate) and cofactor (Mg^{2+}) and higher ammonia levels in the experimental system had no significant toxic effect on the cells, or that this effect was somewhat counteracted by the positive effect of glutamine supplementation.

Monoclonal Antibody Production Analysis

An anti-insulin autoantibody ELISA was performed to quantify monoclonal antibody production in the experimental and control systems. Samples of culture medium from both spinner flasks were collected at the same time as medium transfers. Three dilutions, 1:1, 1:100 and 1:10,000, were employed. The 1:1 dilution yielded absorbance values above the optimal linear range, and were therefore unreliable. Both the 1:100 and 1:10,000 dilutions yielded responses in the linear region, and both followed

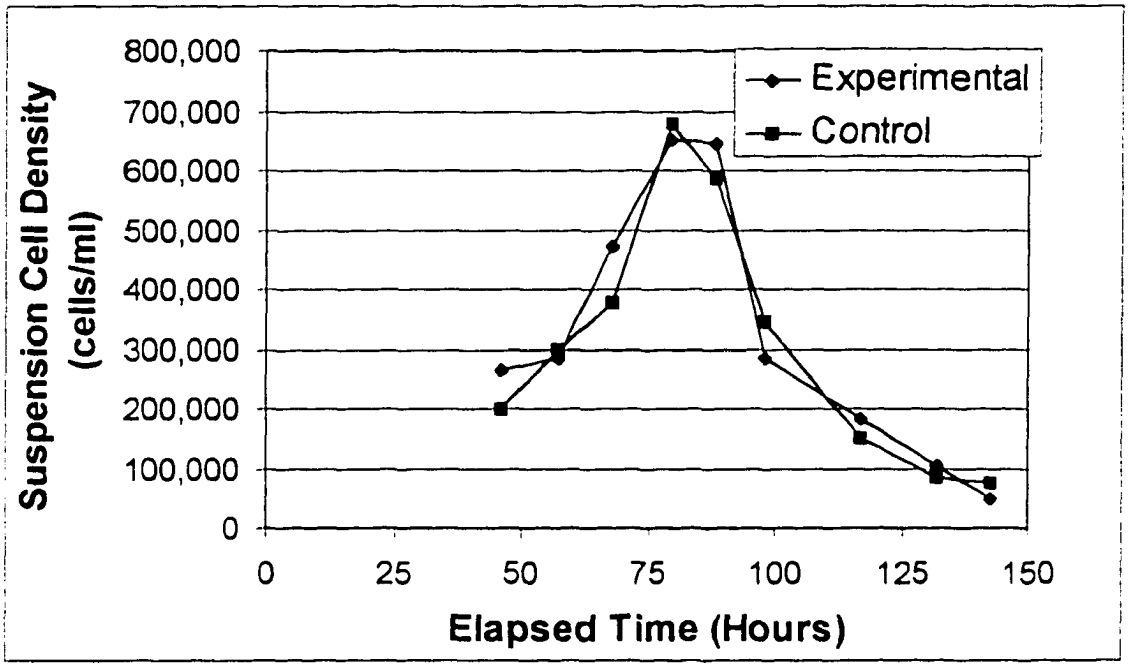

Figure 4.23 Final bioreactor experiment: suspension cell density over time.

trends of increasing MAb concentration with time. However, the 1:10,000 dilution indicated a substantial increase in MAb concentration in the experimental system prior to the first enzymatic conversion, whereas the 1:100 indicated a large increase coinciding with the first enzymatic conversion. Since the experimental and control flasks were treated identically until enzymatic conversion was initiated, the behavior suggested by the 1:10,000 MAb concentration profiles are highly suspect. The two 1:10,000 replicates for the experimental system also demonstrated substantially different trends, and the MAb concentration increase prior to enzymatic conversion start-up was only observed for one of them. These differences could be attributed to the difficulties in pipetting the small volumes required for the 1:10,000 dilution. Note that ELISA assays are often highly

sensitive to such errors. Results from the 1:100 dilution were predominantly more consistent and therefore used in this analysis.

Monoclonal antibody concentration profiles for both systems are presented in Figure 4.24. As the figure indicates, both have similar MAb production prior to 65 hours. The antibody concentration in the experimental system increased significantly between 65 and 95 hours. This interval corresponds to the period immediately after the first batch of glutamine-enriched medium was returned to the experimental culture flask from the reaction chamber, and represents the first point at which differences could be attributed to the glutamine synthetase reaction. As Table 4.8 indicates, the experimental antibody concentration increase is 247% greater than the control increase during this period, and 37% greater between 65 and 130 hours. Between 95 and 130 hours, the two systems once again exhibit similar antibody concentration increases. Glutamine produced in experimental reaction batches 4 and 6 yielded no apparent increase in antibody production. This is likely due to fact that, as displayed in Figure 4.23, the cell culture was in rapid decline during this period, and its viable cell density had already dropped markedly.

Figure 4.24 indicates a drop in antibody concentration within the experimental system at 140 hours. This is likely due to measurement difficulties with the ELISA, since antibodies do not degrade this quickly.

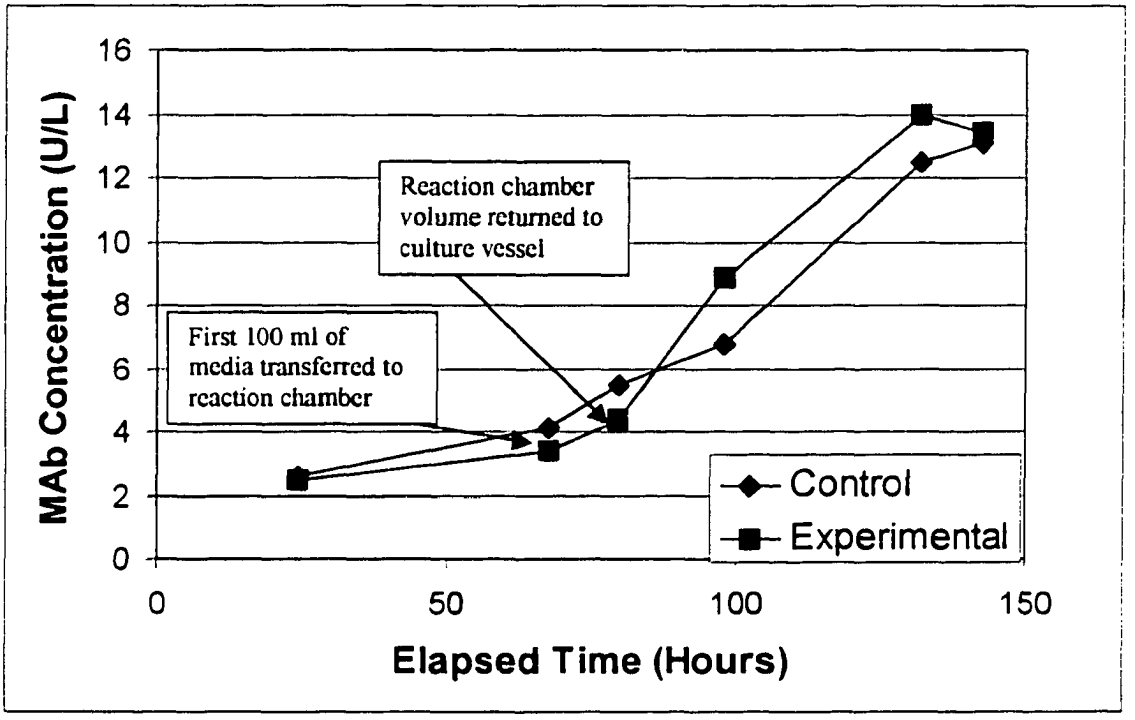

Figure 4.24 Final bioreactor experiment: anti-insulin autoantibody ELISA results, 1:100 dilution.

			MAb Production Rate (U/L-hr)		
Starting Time (Hours)	Ending Time (Hours)	Elapsed Time (Hours)	Control	Experimental	Rate Improvement
65	95	18.5	0.07	0.24	247%
65	130	52.25	0.13	0.18	37%

Table 4.8 Final bioreactor experiment: MAb production rate analysis.

Chapter 5 -- Conclusions

Reactant Effects on Glutamine Synthetase Activity

Based on experimental results, the glutamate concentration appears to have the greatest effect on soluble enzyme initial ammonia conversion rate. This positive effect is consistent with previously published results (Segal and Stadtman, 1972). The second greatest effect on conversion rate was exhibited by Mg^{2+}. Glutamine, ATP and ammonia had small effects. Glutamate also had the greatest positive effect on total ammonia conversion, but here was followed by ATP and ammonia. The effects on Mg^{2+} and glutamine were small, likely within experimental error.

Reaction Equilibrium Analysis

The evaluation of estimated final concentrations of the enzymatic half-reaction

$$NH_3 + Glu \rightarrow Gln$$

yielded an average K_{eq} of 2.13 for experimental treatments with non-limiting ATP. Actual calculated K_{eq}'s ranged from 1.40 to 2.86. This K_{eq} yields a percent ammonia conversion which is substantially higher than obtained experimentally, thus suggesting that conversions are limited by factors other than equilibrium, such as ATP degradation.

Spontaneous ATP Hydrolysis

Significant spontaneous degradation of ATP was observed immediately upon dissolution. Based on observed reaction endpoints, approximately 35% of initial added

ATP underwent spontaneous hydrolysis, and was therefore unavailable for the enzymatic conversion reaction. Titrations of water with ATP/ Mg^{2+} solutions illustrated a clear relationship between the extent of spontaneous hydrolysis and Mg^{2+}:ATP ratio in solution.

Enzyme Activity in Culture Media

1.8 mM Ca^{2+} reduced soluble glutamine synthetase activity by 66%. This sizeable inhibitory effect is consistent with previously published results and indicates that Ca^{2+}-containing culture media, such as DMEM, are generally undesirable for use with *in vitro* glutamine synthetase ammonia conversion. The enzyme activity observed in S-MEM, a Ca^{2+}-free medium, was higher than that observed in bicarbonate buffer. When 10% horse serum was added to S-MEM, a 15% decline in enzyme activity was observed, partly due to Ca^{2+} in the serum.

Immobilization of Glutamine Synthetase

Residual activities observed for glutamine synthetase immobilized via covalent binding to CNBr-activated sepharose beads varied considerably with the immobilization method used. A protocol incorporating rinsing with a pH 4 solution only yielded 2.5% residual activity. When this solution was replaced by a pH 7 buffer, residual activity of approximately 8% was observed. Since glutamine synthetase has been observed to exhibit optimal activity at alkaline or neutral pH's, this dramatic difference is likely due to a chaotropic effect of the pH 4 solution. Sepharose-immobilized glutamine synthetase exhibited near-linear degradation of activity over time at 37 °C, with a half-life of

approximately 24 days. This thermostability represented an improvement over soluble enzyme of approximately 45%.

Glutamine synthetase entrapped within κ-carrageenan beads yielded no measurable conversion activity, and enzyme-containing beads had very poor mechanical stability, degrading within hours in solution.

Enzyme encapsulated within dialysis tubing yielded residual activity of 100%, and exhibited thermostability at 37 °C similar to soluble enzyme, with a half-life of approximately 13.3 days. This behavior is not unexpected due to the nonspecific nature of the immobilization procedure.

Hybridoma Sensitivity To Reactants

Of the four enzyme reactants studied, 10 mM ATP had by far the largest negative effect on maximum hybridoma cell density, at approximately 700,000 cells/ml, which represents approximately 58% of the control treatment maximum density. 3 mM ammonia, 8 mM Mg^{2+}, and 10 mM glutamate all had much smaller, but significant negative effects.

Immobilized Glutamine Synthetase Within an Active Hybridoma Cultivation

Ammonia conversion within the reaction chamber of the experimental system was less than expected based on preliminary experiments; the largest amount of ammonia converted was 0.4 mM in the first batch. These poor results were likely due to a combination of two factors: clogging of the enzyme-encapsulating dialysis tubing by

suspension cells, and the precipitation of Mg^{2+} in solution prior to use. In light of these problems, ammonia concentrations in the experimental system actually rose higher than in the control system. One possible explanation of this effect is that the experimental enzymatic conversion provided enough glutamine supplementation to keep the hybridomas metabolically active, and the increasing ammonia concentrations observed were due to the metabolism of additional amino acids from the culture medium and/or animal serum.

The experimental and control systems exhibited very similar suspension cell density profiles over the entire duration of the experiment. This suggests that either the enzymatic reactants did not accumulate to high enough concentrations to adversely affect the cells, or that this adverse effect was somewhat counteracted by the beneficial effect of glutamine supplementation.

Finally, a comparison of experimental and control antibody concentration profiles illustrated a marked increase in experimental antibody concentration corresponding to the first glutamine supplementation. The experimental antibody concentration during this period was 247% of the control system increase. Later glutamine supplementation did not provide any significant beneficial effects, possibly since both systems had reached the late death phase of their culture life cycles by this time.

Overall, although ammonia conversion and glutamine supplementation was less than expected, immobilized glutamine synthetase shows promise as a means of improving hybridoma cell culture and decreasing the cost of MAb production.

Chapter 6 – Future Directions

The project presented here was intended as a "proof of concept" of the potential of immobilized glutamine synthetase as a tool for enhancing *in vitro* hybridoma cell culture. As such, this project was somewhat successful; enzymatic glutamine replenishment appears to have resulted in higher MAb concentrations than a parallel non-replenished system. However, the enzymatic conversion system as described and employed in this project has substantial limitations, and at this time is likely not a cost-effective alternative to more traditional ammonia reduction techniques (e.g., media perfusion). To improve upon the enzymatic conversion system and make it more attractive for industrial use, enhancements should be investigated in four primary areas: alternative immobilization methods, alternative culture media, ATP replenishment, and system automation.

Alternative Immobilization Methods

Due to the poor residual enzyme activities offered by covalent binding to CNBr-activated sepharose beads (approx. 7%), and by entrapment within κ-carrageenan gel beads (0%), glutamine synthetase encapsulated within dialysis tubing was employed in the final bioreactor system. As previously described, enzymatic ammonia conversion in this system was less complete than expected, and the primary suspected culprit for this problem was clogging of the dialysis tubing with suspension hybridoma cells. This is not a surprising result, as similar membrane fouling with suspension cells was reported by Nayve and coworkers (1994) in their ammonia removal system.

In light of this difficulty, enzyme immobilization methods which do not employ membranes offer an obvious advantage, and warrant further consideration. The poor conversion activities observed for glutamine synthetase immobilized on CNBr-activated sepharose and in κ-carrageenan gel should not be interpreted as failures of covalent binding and entrapment methods as a whole. The covalent binding method used in this study, which uses cyanogen bromide as an activator, uses relatively toxic reagents and yields bonds with relatively poor stability (Scouten, 1987). Covalent activation methods with lower toxicity and better bond stability, such as trezyl chloride and benzoquinone, should also be investigated. Regarding entrapment, the poor results observed with carrageenan gel beads were likely due to cation exchange between NH_4^+ in solution and K^+ in the gel matrix and subsequent enzymatic conversion of this gel-entrapped NH_4^+, which damaged the integrity of the gel matrix. Entrapment methods which do not require cation hardening would likely not present this problem, and may offer a good alternative to membrane encapsulation.

Alternative Culture Media

As discussed in Chapter 4, DMEM, an effective and widely used hybridoma culture medium, was found to be a poor environment for glutamine synthetase conversion, primarily due to its Ca^{2+} content. S-MEM was therefore used in subsequent experiments. However, DMEM is a richer medium than S-MEM, and in an earlier unpublished study performed by our laboratory group, DMEM was found to support more rapid cell growth and higher cell densities than S-MEM. To support both high

hybridoma cell densities and rapid glutamine synthetase activity, another culture medium should be employed. This medium may simply be DMEM without Ca^{2+}, or may also exclude some other enzyme-inhibiting substrates which have not yet been identified.

Animal blood serum was also found to be inhibitory to glutamine synthetase activity. This effect is likely due at least partially to the Ca^{2+} content of serum, but may also be attributable to other as of yet unidentified serum components. This suggests that immobilized glutamine synthetase would be particularly effective in serum-free media, from which Ca^{2+} and other inhibitory agents (if any) could easily be omitted. Studies of enzyme activity in such media could be undertaken to test this hypothesis, and possibly to identify other enzyme-inhibitory serum components.

ATP Replenishment

One of the primary limitations of the glutamine synthetase ammonia-conversion system described in this study is its reliance on the continued addition of ATP to the culture medium to support conversion activity. This requirement is problematic for two reasons: ATP is quite expensive, and, as shown in Chapter 4, it is toxic to hybridoma cells at relatively low concentrations. One potential method for reducing the need for ATP replenishment is through enzymatic ATP synthesis. Steinberg-Yfrach and coworkers (1998) have developed such a method, utilizing a proton gradient and the enzyme ATP synthase to synthesize ATP from ADP. This method may prove applicable in tandem with the glutamine synthetase system studied here; if so, this could reduce cost and improve feasibility for use in industrial hybridoma cell cultivation.

System Automation

The glutamine synthetase-incorporating bioreactor system as described in this study required substantial human labor to operate and maintain. The enclosed system used in the test run and represented in Figure 3.2 offers some improvement over the entirely manual fluid transfer methods used in the final bioreactor experiment, but still requires manual pump operation and valve turning, and off-line ammonia measurement. Substantial automation opportunities exist to make this system much less labor intensive.

For example, a separate unpublished study performed by our laboratory group demonstrated the ability to provide accurate measurements of ammonia, glutamine and glutamate (as well as lactate and glucose) in culture medium via near-infrared (NIR) spectroscopy. An FTIR spectrometer could be incorporated in a bioreactor system to provide real-time ammonia, glutamine and glutamate concentrations. This information could be used to automatically trigger media transfer (e.g. from the culture chamber to the reaction chamber and vice versa), and reactant supplementation of optimal volumes and ratios.

Appendix 1

DMEM and S-MEM Components Lists

SIGMA Σ
CHEMICAL COMPANY

DULBECCO'S MODIFIED EAGLE'S MEDIUM (DME)
With 4500 mg/L Glucose, 110 mg/L Sodium Pyruvate, L-Glutamine
Without Sodium Bicarbonate
Product No. D-6780
Store at 2-8°C
HYBRI-MAX®

DULBECCO'S MODIFIED EAGLE'S MEDIUM [DME]

PRODUCT DESCRIPTION

Many modifications of Eagle's Medium have been developed since the original formulation appeared in the literature. Among the most widely used of these modifications is Dulbecco's Modified Eagle's Medium (DME). DME is a modification of Minimum Essential Medium Eagle (MEM) that contains a higher concentration of amino acids and vitamins, as well as additional supplementary components. The original DME formula contains 1000 mg/L of glucose and was first reported for culturing embryonic mouse cells. A further alteration with 4500 mg/L glucose has proven to be optimal in cultivating certain cell types.

DULBECCO'S MODIFIED EAGLE'S MEDIUM [DME], Product No. D-6780 is one of the cell culture media available from Sigma. The selection of a nutrient medium is strongly influenced by 1] type of cell, 2] type of culture [monolayer, suspension, clonal] and 3] degree of chemical definition necessary. It is important to review the literature for recommendations concerning medium, supplementation and physiological parameters required for a specific cell line.

COMPONENTS	g/L
Calcium Chloride•$2H_2O$	0.265
Ferric Nitrate•$9H_2O$	0.0001
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.4
Sodium Chloride	6.4
Sodium Phosphate Monobasic (anhydrous)	0.109
L-Arginine•HCl	0.084
L-Cystine•2HCl	0.0626
L-Glutamine	0.584
Glycine	0.03
L-Histidine•HCl•H_2O	0.042
L-Isoleucine	0.105
L-Leucine	0.105
L-Lysine•HCl	0.146
L-Methionine	0.03
L-Phenylalanine	0.066
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016
L-Tyrosine•2Na•$2H_2O$	0.10379
L-Valine	0.094
Choline Chloride	0.004
Folic Acid	0.004
myo-Inositol	0.0072
Niacinamide	0.004
D-Pantothenic Acid (hemicalcium)	0.004
Pyridoxal•HCl	0.004
Riboflavin	0.0004
Thiamine•HCl	0.004
D-Glucose	4.5
Phenol Red•Na	0.0159
Pyruvic Acid•Na	0.11

REAGENT
For In Vitro Diagnostic Use

PREPARATION INSTRUCTIONS

Powdered media are extremely hygroscopic and should be protected from atmospheric moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated solution of medium is not recommended as precipitates may form.

Supplements can be added prior to filtration or introduced aseptically to sterile medium. The nature of the supplement may affect storage conditions and shelf life of the medium.

1. Measure out 90% of final required volume of water. Water temperature should be 15-20°C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. Do NOT heat.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
4. To the solution in step 3, add 3.7 g sodium bicarbonate or 49.3 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Aseptically dispense medium into sterile container.

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MINIMUM ESSENTIAL MEDIUM EAGLE [S-MEM] FOR SUSPENSION CULTURE

With L-glutamine Without Sodium Bicarbonate
Product No. M-4767
Store at 2-8°C

PRODUCT DESCRIPTION

Minimum Essential Medium (MEM), developed by Harry Eagle, is one of the most widely used of all synthetic cell culture media. Early attempts to cultivate normal mammalian fibroblasts and certain subtypes of HeLa cells revealed that they had specific nutritional requirements that could not be met by Eagle's Basal Medium (BME). Subsequent studies using these and other cells in culture indicated that additions to BME could be made to aid growth of a wider variety of fastidious cells. MEM, which incorporates these modifications, includes higher concentrations of amino acids. MEM has been used for cultivation of a wide variety of cells grown in monolayers. Optional supplementation of non-essential amino acids to the formulations that incorporate either Hanks' or Earle's salts has broadened the usefulness of this medium. The formulation has been further modified by optional elimination of calcium to permit growth of cells in suspension culture.

MINIMUM ESSENTIAL MEDIUM EAGLE [S-MEM] FOR SUSPENSION CULTURE, Product No. M-4767 is one of the cell culture media available from Sigma. The selection of a nutrient medium is strongly influenced by 1] type of cell, 2] type of culture [monolayer, suspension, clonal] and 3] degree of chemical definition necessary. It is important to review the literature for recommendations concerning medium, supplementation and physiological parameters required for a specific cell line.

COMPONENTS	g/L
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.4
Sodium Chloride	6.8
Sodium Phosphate Monobasic (anhydrous)	1.22
L-Arginine•HCl	0.126
L-Cystine•2HCl	0.0313
L-Glutamine	0.292
L-Histidine•HCl•H_2O	0.042
L-Isoleucine	0.052
L-Leucine	0.052
L-Lysine•HCl	0.0725
L-Methionine	0.015
L-Phenylalanine	0.032
L-Threonine	0.048
L-Tryptophan	0.01
L-Tyrosine•2Na•2H_2O	0.0519
L-Valine	0.046
Choline Chloride	0.001
Folic Acid	0.001
myo-Inositol	0.002
Niacinamide	0.001
D-Pantothenic Acid (hemicalcium)	0.001
Pyridoxal•HCl	0.001
Riboflavin	0.0001
Thiamine•HCl	0.001
Glucose	1.0
Phenol Red•Na	0.011

REAGENT
For In Vitro Diagnostic Use

PREPARATION INSTRUCTIONS

Powdered media are extremely hygroscopic and should be protected from atmospheric moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated solution of medium is not recommended as precipitates may form.

Supplements can be added prior to filtration or introduced aseptically to sterile medium. The nature of the supplement may affect storage conditions and shelf life of the medium.

1. Measure out 90% of final required volume of water. Water temperature should be 15-20°C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. Do NOT heat.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
4. To the solution in step 3, add 2.2 g sodium bicarbonate or 29.3 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Aseptically dispense medium into sterile container.

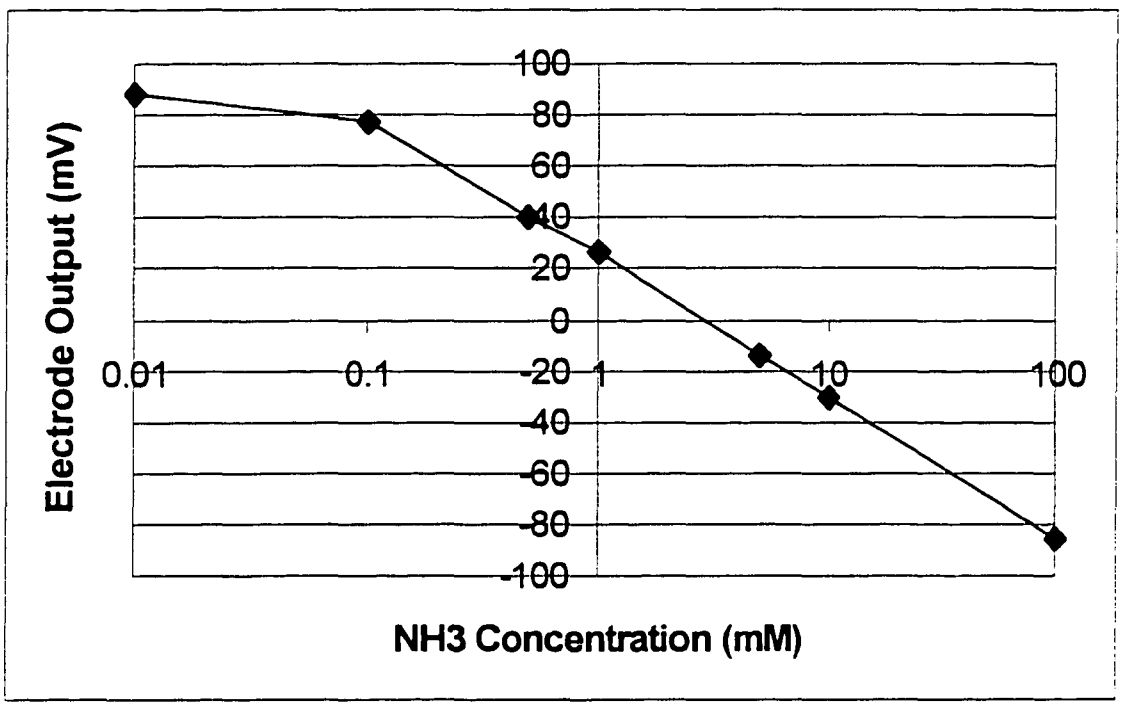
Appendix 2

Supplies, Vendors and Part Numbers List

Item Description	Vendor	Part Number
Cell Culture Supplies		
Mouse-mouse hybridoma cells	ATCC	CE9H9
DMEM medium	Sigma	D-6780
S-MEM medium	Sigma	M-4767
Horse serum	Sigma	H-1270
Penicillin-streptomycin solution	Sigma	P-7539
Antibiotic/antimycotic solution	Sigma	A-9909
E. coli glutamine synthetase	Sigma	G-3144
Enzyme/Cell Immobilization Materials		
CNBr-activated sepharose 6MB	Sigma	C-9267
Carrageenan - kappa	Sigma	C-1263
Dialysis tubing	Spectra/Por	132,678
Dialysis tubing closures	Spectra/Por	132,736
Agarose type IX-A	Sigma	A-2576
Measurement Equipment and Supplies		
Ammonia-sensing electrode	Orion	9512BN
ISA solution - ammonia-sensing electrode	Orion	951211
Insulin autoantibody assay kit	ALPCO	008-10-1121-01
Bioreactor System Equipment		
Flexible tubing	Cole-Parmer	95609-48
Reversible pump	Cole-Parmer	77120-62
Three-way valves	Cole-Parmer	30600-04
Male luer-lock fittings	Cole-Parmer	30504-10
Female luer-lock fittings	Cole-Parmer	06359-37

Appendix 3

Ammonia Electrode – Sample Calibration Curve


Electrode Output (mV)
100
80
60
40
20
0
-20
-40
-60
-80
-100
0.01
0.1
1
10
100
NH3 Concentration (mM)

Appendix 4

Curve-Fitting Procedure for Soluble Enzyme Experiment

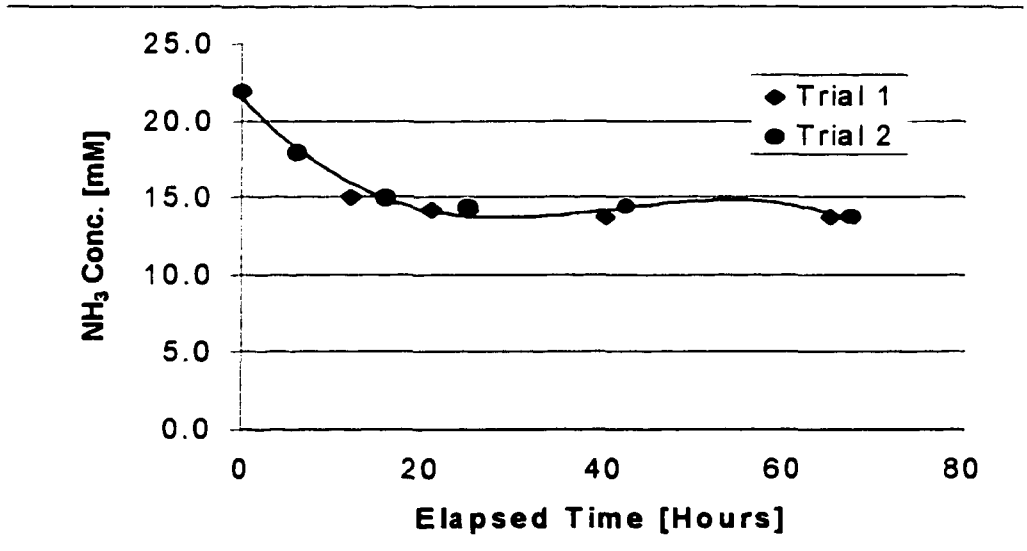
The objective of the curve fitting performed for the experiment which evaluated soluble glutamine synthetase activity in buffer was strictly to provide a regression of the experimental data, not a correlation. In other words, curves were sought that fit the observed data well, and no attempt was made to develop a generally applicable model. The resulting curves were used to interpolate 17-hour and 60-hour ammonia concentrations from which the initial ammonia conversion rates and total ammonia conversions could be calculated.

As described in Chapter 4, data from the two replicates of each experimental treatment were normalized to a common initial ammonia concentration by multiplying each data point from one of the replicate data sets by a common factor. Justification for this action relies on the assumption that the enzymatic conversion activity exhibits similar behavior at two slightly different (generally 1mM or less) initial ammonia concentrations. In hindsight, this assumption seems quite reasonable, since, as presented in Tables 4.1 and 4.2, a 10 mM initial ammonia concentration difference only resulted in an initial ammonia conversion rate effect and a total ammonia conversion effect representing 2% and 10% of the respective mean treatment effects.

The normalized replicate data sets were combined to provide 10 composite data sets (ammonia concentration in mM versus elapsed time in hours), and optimized curves were fit separately to each set using the "LINEST" array function in Microsoft Excel 97. As previously discussed, curves of the form $y = Ax + Bx^2 + Cx^3 + D$ were first fit to each treatment, but introduced curve-fitting artifacts. These artifacts took the form of "humps" in the curves, generally near the 60-hour point. The figure below presents the third-order

curve generated for the data from Treatment 2. This curve suggests that an ammonia concentration increase occurred during the latter portion of the experiment, followed by an inflection point near 55 hours, and a subsequent decrease. This behavior is not supported by any experimental observations, and interpolations from this curve yielded misleading 60-hour total ammonia conversion values.

Curves of the form $y = Ax + Bx^{1/2} + C$ were next evaluated. This somewhat unusual form was chosen strictly due to the observed shape of the data, which is roughly parabolic with the form $x = Ay^2 + By + C$. Curves of this form fit to the data from each experimental treatment using the "LINEST" function provided high R^2 values without artifacts in the regions of 17 or 60 hours, and were therefore used for all interpolations for this experiment.



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