Effect of Extracellular Glutamine Concentration on Primary and Secondary Metabolism of a Murine Hybridoma: An In Vivo ^{13}C Nuclear Magnetic Resonance Study

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Abstract: The effect of changes in extracellular glutamine level on metabolism of a murine hybridoma was examined with in vivo nuclear magnetic resonance (NMR) spectroscopy. Cells were cultured in a hollow-fiber bioreactor at high cell density to allow intracellular metabolite levels to be determined on a metabolically relevant time scale. Steady infusions of [1-^{13}C] glucose were used to label glycolytic and tricarboxylic acid cycle intermediates, which permitted continuous monitoring with NMR spectroscopy during changes in environmental glutamine level. Samples of the extracellular medium were also analyzed to determine the effect of glutamine on other metabolites associated with primary and secondary metabolism. The changes in glutamine concentration had several effects on primary and secondary metabolism, depending on the rate the changes were made. For a brief reduction in feed glutamine concentration from 4 to 0 m*M* (which produced a rapid change from 0.67 to ~0 m*M* in residual glutamine), large changes were observed in the rate of consumption of metabolites normally associated with energy production. Antibody synthesis was strongly stimulated and nitrogen metabolism was significantly altered. For a more prolonged reduction from 2.4 to 1.2 m*M* (which produced a slower reduction from 0.30 to 0.08 m*M* in residual glutamine), much smaller changes were observed even though the concentration of glutamine at the reduced feed level was very low. Energy metabolism did not appear to be limited by glutamine at 0.08 m*M*, which suggests that significant futile cycling may occur in energy producing pathways when excess glucose and glutamine are available. However, this concentration of extracellular glutamine appeared to affect some anabolic pathways, which require amino groups from glutamine. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **57:** 172–186, 1998.

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INTRODUCTION

High rates of glutamine consumption are commonly observed in hybridomas and many other cell types exhibiting high rates of proliferation. Glutamine is catabolized through the glutaminolytic pathway to pyruvate (McKeehan, 1986), which can subsequently be converted to either lactate, or carbon dioxide and water in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Glutamine often serves as a major energy source during rapid proliferation (Miller et al., 1989; Reitzer et al., 1979) and provides amine groups for the synthesis of purines and pyrimidines (McKeehan, 1986). Little is known about the regulation of glutaminolysis and greater understanding of this process could lead to improvements in monoclonal antibody production; previous results from our laboratory have indicated that an abrupt change in environmental glutamine level can markedly affect specific antibody production. Moreover, insights into the regulation of glutamine consumption can facilitate the direction of future genetic engineering approaches (Bell et al., 1995; Brown et al. 1992) to improving monoclonal antibody productivity.

The effects of environmental glutamine levels on the rates of primary metabolism, cell growth, and antibody production have been examined for a number of hybridoma lines (Miller and Blanch, 1991). Initial rate studies conducted in batch culture have demonstrated that the glutamine concentration for half-maximal growth rate (K_{gln}) is typically in the range of 0.09 to 0.15 m*M* (Flickinger et al., 1992; Glacken et al., 1988; Jeong and Wang, 1995). At levels well above K_{gln} (1.5–8 m*M*), growth rates are inde-

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pendent of glutamine concentration (Glacken et al., 1988; Miller et al., 1988), which is consistent with simple Monod growth kinetics. However, at these higher levels, the specific rate of glutamine consumption (q_{gln}) is often increased (Dalili et al., 1990; Jeong and Wang, 1995; Miller et al., 1988), suggesting that glutamine consumption kinetics are complex.

More accurate measurements on the effect of glutamine concentration have been conducted in steady-state chemostatic culture. Miller and co-workers compared metabolic rates for AB2-143.2 hybridomas grown at residual glutamine concentrations of 0.2 and 3.0 m*M,* in the presence of excess glucose (Miller et al., 1989). At the higher glutamine concentration, q_{gln} was higher and was associated with lower specific rates of glucose consumption (q_{gluc}) and lactate production (q_{lac}). Ammonium and alanine specific productivity (q_{NH_3} and q_{ala}, respectively) were both higher also. For another (unspecified) hybridoma line, Schmid and Keller determined metabolic rates for extracellular glutamine concentrations ranging from 0.24 to 1.69 m*M* (Schmid and Keller, 1992). Over this range, the specific rates of growth and antibody formation (q_{Ab}) were relatively independent of the glutamine concentration. Further, q_{gln}, q_{NH_3} and q_{ala} generally increased with increasing glutamine concentration. These results are generally consistent with those observed by Miller et al. However, unlike Miller et al., Schmid and Keller observed increased q_{gluc} and q_{lac} with increasing glutamine concentration.

Regulation of glutaminolysis in hybridomas has been examined in unsteady-state suspension culture experiments by Miller et al. (1989) with step changes in the extracellular glutamine concentration. The immediate metabolic responses (<24 h) to such changes depended on the initial glutamine concentration. In the presence of excess glucose, a step change from 0.2 to 3.0 m*M* glutamine increased q_{gln} by approximately 50% without producing a significant change in either q_{gluc}, q_{lac} or the specific consumption rate of oxygen (q_{O_2}). However, q_{NH_3} and q_{ala} increased substantially. No significant change in viable cell concentration was observed. The lack of increase in cell growth suggests that the energy available from the higher glutaminolytic flux was not used for energy production but was probably wasted by futile cycling (Newsholme et al., 1992).

Miller et al. (1989) also observed that a step change from approximately 0 to 0.9 m*M* in extracellular glutamine (with glucose present in excess) produced a marked increase in consumption of glucose, glutamine and oxygen, and production of ammonium, alanine and lactate. The cell concentration also increased significantly approximately 12 h after the addition of glutamine. These results indicate that cellular metabolism was anabolically limited by the availability of amino groups at the low initial glutamine concentration.

In a previously reported hollow-fiber bioreactor study, we observed that reducing the extracellular glutamine concentration from approximately 1.2 to 0.3 m*M* had little effect on glucose metabolism of 4A2 hybridomas (Sharfstein et al., 1994). We observed no significant change in the rate of glycolysis, as indicated by a lack of change in levels of ^{13}C-labeled metabolites monitored with in vivo NMR spectroscopy. In addition, the flux of glucose-derived carbon through pyruvate dehydrogenase into the TCA cycle appeared to be unaffected since no change was observed in [4-^{13}C] glutamate labeling. (^{13}C labeling in glutamate directly reflects ^{13}C labeling in α-ketoglutarate because the two compounds are in equilibrium (Chance et al., 1983)). These findings suggest that environmental glutamine levels below 0.3 m*M* may be necessary to produce significant changes in the glycolytic and pyruvate dehydrogenase fluxes.

The goal of this work was to examine the effects of changes in extracellular glutamine concentration on metabolism of 4A2 hybridomas at levels lower than those used in our previously reported work. In vivo NMR spectroscopy was used to monitor levels of intracellular and extracellular metabolites during a large, brief reduction and a more prolonged reduction in glutamine concentration. Non-NMR methods were also used to determine the concentration of extracellular primary metabolites and amino acids during the changes.

MATERIALS AND METHODS

Cell Line and Culture Conditions

Hybridoma cell line 4A2 (formerly X-D) from Xoma Corporation (Berkeley, CA) was used for this work. It was derived from an NS1 myeloma parent and produces an IgG type antibody. Characteristics of this organism determined in continuous suspension culture have been reported by Hiller et al. (1991, 1993, 1994). This cell line was found to be mycoplasma free prior to use in this work (testing performed by Xoma Corporation).

The basal culture medium was serum free 1:1 DME:F12 (Dulbecco's Modified Eagle's: Ham's F12) medium containing no glucose or glutamine (JRH Biosciences, Lenexa, KS). The concentrations of glucose (either labeled or unlabeled) and glutamine added to the medium were 17 and 4 m*M,* respectively. The medium was supplemented with 1× BME (Basal Medium Eagle) amino acids (JRH Biosciences), sodium selenite: 3.46 μg/L, ethanolamine-HCl: 1.93 mg/L, sodium bicarbonate: 1.68 gm/L, HEPES: 1.20 gm/L, sodium HEPES: 3.36 gm/L, albumin: 100 mg/L (bovine, essentially fatty acid free, Sigma, St. Louis, MO), and transferrin: 30 mg/L (bovine, iron poor, Miles Labs, Kankakee, IL). The sodium chloride content of the medium was reduced to 5.79 gm/L (from 7.00 gm/L) to reduce the total osmolite level. The medium pH was adjusted to 7.7 with 4N potassium hydroxide prior to filter sterilization. All medium components were tissue culture grade. Antibiotics were not used, since they can mask mycoplasma infection.

For culture maintenance and inoculum development, cells were grown in T-75 or T-150 flasks from Corning (Fisher Scientific, Santa Clara, CA). The flasks were kept in

a humidified incubator maintained at 37°C, with a 5% CO_2/ 95% air atmosphere.

Hollow Fiber Bioreactor Apparatus

The hollow fiber bioreactor used was custom manufactured by Microgon, Inc. (Laguna Hills, CA). It was identical to the reactor described in a previous report (Mancuso et al., 1994) with the exception that the working length was 80 mm. The maximum diffusion distance from the outer fiber membrane to the midpoint between fibers was on average 65 microns or approximately 4 cell diameters. Previous results have indicated that 4A2 hybridoma metabolism in this reactor is not limited by oxygen transport if the lumen concentration at the reactor inlet is near air saturation, and the flow rate through the reactor is high (Mancuso, 1993).

The apparatus used with the hollow fiber bioreactor was similar to that described previously (Fernandez et al., 1990). The reactor was operated in a recycle mode at a high recycle ratio of 350:1 (recycle : feed) to minimize axial gradients. Medium was circulated through the reactor with a gear pump (Micropump series 1840, Concord, CA). Nutrient levels in the recycle stream other than oxygen were maintained at steady state with continuous fresh medium addition and spent medium removal from a recycle vessel with a peristaltic pump (Rabbit®, Rainin, Emeryville, CA). The feed medium was maintained at 4°C throughout the experiment and pumped to the system at a rate of 28 ± 1 mL/min.

Dissolved oxygen (DO) was monitored on both sides of the reactor with steam sterilizable probes (Ingold, Lexington, MA). Signals from the DO monitors (Instrument Labs Model IL 531 Lexington, MA) were recorded continuously with an 8088-based IBM compatible computer equipped with two IBM data acquisition boards (IBM, Boca Raton, FL) (Mancuso, 1993). The computer continuously calculated the oxygen consumption rate (q_{O_2}) from the equation:

$$Q_{O_2} = F_{recirc}\,[O_2]_{sat}\,(DO_{in} - DO_{out})/100 \qquad (1)$$

where: Q_{O_2} = total oxygen consumption rate (mmol/h), DO_{in} = dissolved oxygen level at the reactor inlet, DO_{out} = dissolved oxygen level at the reactor outlet (both as a % of saturation with air), F_{recirc} = volumetric flow rate of medium through the reactor (L/h), and $[O_2]_{sat}$ = the concentration of dissolved oxygen in growth medium saturated with air at 37°C. This value was estimated to be 0.20 m*M* (Benson et al., 1976; Schumpe et al., 1978).

pH was monitored at the outlet of the reactor with a steam-sterilizable glass electrode (Ingold, Lexington, MA). The medium oxygen and pH levels were controlled by manually adjusting the gas phase composition in the hollow fiber gas exchange module (Network®, Kinetec Inc., St. Louis, MO), with a N_2, O_2, and CO_2 gas mixer (Matheson 7400 Series, Newark, CA). Control ranges for DO_{in} and pH were 110 ± 10% of saturation, and 7.25 ± 0.07, respectively.

The temperature of the circulating medium was monitored with thermistors (Model YSI 44001A, Yellow Springs Instrument Corp., Yellow Springs, OH) mounted in insulated Pyrex temperature wells. A heated warm water system, controlled with a second 8088-based IBM compatible computer was used to maintain the inlet reactor temperature at 37.0 ± 0.1°C (Fernandez, 1989). Additional details of the hollow fiber bioreactor apparatus are available elsewhere (Mancuso, 1993).

The entire system, less the hollow fiber bioreactor, was steam autoclaved at 121°C for 35 minutes. The bioreactor was sterilized by the manufacturer, and was connected to the rest of the recirculation system in a biological safety cabinet. After filling the system with sterile medium, the reactor was inoculated with 2×10^8 cells grown to early stationary phase. During the initial growth phase (0 to 176 h after inoculation), the medium feed rate was gradually increased from 0 to 28 mL/min.

In Vivo NMR Methodology

The spectrometer used in this work was assembled at the U.C. Berkeley College of Chemistry NMR Facility from commercially available components (Mancuso et al., 1994). The resonant frequency for hydrogen nucleus was 180 MHz. The spectrometer was controlled with a LIBRA pulse programmer (Tecmag Inc., Houston, TX) that was driven by a Macintosh IIci computer (Apple, Cupertino, CA).

A pair of orthogonal Helmholtz coils mounted directly on the reactor were used for pulse transmission and signal reception (Mancuso et al., 1994). The inner coil was broadband tunable, and was used for carbon, sodium, and phosphorous spectroscopy. Sodium and phosphorous spectra were acquired with simple one-pulse sequences. Cell concentrations were determined from sodium spectra and relative ATP concentrations were determined from phosphorous spectra (Mancuso et al., 1990). Carbon spectra of labeled metabolites were acquired with a Distortionless Enhancement by Polarization Transfer sequence (DEPT 45). These spectra were acquired as 600 scan blocks, each collected over a 33 min time span. The methods used for identifying chemical species detected in in vivo spectra and for converting resonance intensities to concentrations were described in a previous report (Mancuso et al., 1994).

Extracellular Metabolites

Samples were withdrawn periodically from the recirculating medium through a line that extended into a biological safety cabinet. They were preserved with 0.02% sodium azide and stored at −10°C for subsequent analyses. Glucose and lactate concentrations were determined with colorometric enzyme assays (Hiller et al., 1991). Amino acid concentrations were determined by high performance liquid chromatography with an automated system (Applied Biosystems Inc., Foster City, CA) (Hiller et al., 1991). Ammonium concentrations were determined with an ion specific probe (Orion, Boston, MA), and antibody concentrations were determined by protein A chromatography (Bio-Rad, Cambridge, MA) with UV detection at 280 nm (Hiller et al., 1991). The

results were used to estimate consumption and production rates from the equation below:

$$Q = (C_{feed} - C_{recirc})\, F_{sys} - V_{sys} \frac{dC_{recirc}}{dt} \qquad (2)$$

In this equation, Q = consumption or production rate of a metabolite (mmol/h), C_{feed} = concentration of metabolite in the feed (mM), C_{recirc} = concentration of metabolite in the recirculating medium (mM), V_{sys} = recirculation system volume (0.180 L), F_{sys} = volumetric feed rate (L/h), and t is time (h).

RESULTS

Growth Profiles

Growth curves during the hollow fiber experiment are shown in Figure 1. ^{23}Na spectroscopy indicated that the cell concentration in the reactor increased with time from 75 h to 175 h and reached a steady state level of approximately 1.6 ($\pm$ 0.1) $\times$ 10^8 cells/mL of extracapillary space (ECS); the level at 310 h was very similar at 1.7 ($\pm$ 0.1) $\times$ 10^8 cells/mL of ECS. ^{23}Na cell concentration measurements were not made between 220 and 290 h because during that time carbon spectra were acquired. The total ATP concentration in the reactor followed a similar pattern. However, it appeared to reach a maximum at about 175 h and subsequently declined slightly by 220 h. A further slight decline (approximately 10%) was observed between 220 h and 290 h.

Typical ^{13}C Spectrum

Infusion of 17 mM [1-^{13}C] glucose in place of all unlabeled glucose was initiated approximately 215 h after inoculation and was continued for the following 72 h. A typical 66 min steady state spectrum from the reactor (acquired 267 h after inoculation) is shown in Figure 2. Very strong resonances were observed for α and β-[1-^{13}C] glucose. These signals predominantly represent extracellular glucose because the concentration of intracellular glucose in transformed cells is low (Fiechter and Gmunder, 1989). The resonance associated with [3-^{13}C] lactate is also very strong; it arises from both intracellular and extracellular lactate, which cannot be distinguished in this spectrum. Other smaller resonances were [2-^{13}C] lactate, [1-^{13}C] fructose-1,6-diphosphate (F-1,6-DP) and [3-^{13}C] of 3-carbon glycolytic intermediates, a group of overlapping resonances corresponding to [1-^{13}C] fructose-6-phosphate (F-6-P) and the -CH_2- groups of the glycerol backbone of triglycerides, [2-^{13}C], [3-^{13}C], and [4-^{13}C] glutamate, several overlapping resonances corresponding to the -CH_2- groups in the center of fatty acyl chains, [3-^{13}C] pyruvate, and [3-^{13}C] alanine. The glutamate-3 and -4 resonances contain contributions from coresonant fatty acyl groups (Cohen, 1987). A description of how labeled carbon is incorporated into each of these metabolites was presented previously (Mancuso et al., 1994). Five distinct resonances were also observed for the naturally abundant ^{13}C in HEPES. These resonances and some of the lipid resonances were detectable before the infusion of [1-^{13}C] glucose.

Response to a Brief Reduction in Feed Glutamine Concentration from 4 to 0 mM

The effects of a large reduction in feed glutamine concentration were examined during a brief experiment initiated 240 h after inoculation. The feed glutamine level to the system was switched from 4.0 mM to 0 mM and was maintainedlevelt3(levethlt3(levevel)-46lever)-345eve4345eveh(o).345eveDingl
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0.67 m*M* to essentially 0 m*M* in approximately 4 h. An 80% reduction in q_{gln} and a 55% increase in q_{gluc} were observed within one h of the feed change. The residual glucose concentration dropped to below 1 m*M* as indicated by the off-line total glucose data. The concentrations of glucose determined in vivo for the reactor were less than those determined off-line because the in vivo measurements are effectively an average of the intracellular concentration [essentially zero (Fiechter and Gmunder, 1989)] and the extracellular concentration. A slightly delayed increase in the [3-^{13}C] lactate and [3-^{13}C] alanine concentrations was observed; similar changes were observed for total lactate and alanine. The increase in q_{lac} and q_{ala} was approximately 20%. The level of [3-^{13}C] lactate determined in vivo is much lower than the level of total lactate because only 40% (approximately) of the lactate is labeled when 4A2 cells are grown on [1-^{13}C] glucose (Mancuso et al., 1994). The level of [3-^{13}C] alanine determined in vivo is similar to the total level determined off-line because although only 40% of the alanine is labeled, the intracellular concentration of alanine is very high, about 15–19 m*M* (Mancuso et al., 1994). The oxygen consumption rate did not change immediately after the reduction in glutamine level, but after a 3 h delay, it dropped from approximately 0.11 to 0.095 mmol/10^9 cells-h. No change was observed in the concentration of [4-^{13}C] glutamate, a predominately intracellular metabolite (Mancuso et al., 1994).

The reduced glutamine concentration also resulted in a doubling of q_{Ab} after a slight delay. q_{NH_3} approximately doubled initially from 0.026 to 0.053 mmol/10^9 cells-h, but the increase was only transient. This increase was unexpected since glutamine is the primary source for ammonium production. Some of the additional ammonium was produced by increased consumption of other amino acids (Fig. 5, Table I). Specifically, the consumption rate of asparagine, aspartate, isoleucine, leucine, lysine, valine, phenylalanine, threonine, and tyrosine increased significantly. However, these increases were not enough to offset the reduced consumption of amino groups from glutamine; the results in Table I show that the total consumption rate of amino groups (from amino acids) dropped from 0.118 to 0.059 mmol/10^9 cells-h. Glycine, which is normally consumed at a very low rate by 4A2 hybridomas, was produced transiently in response to the reduction in the residual glutamine concentration.

On restoring glutamine to the feed at a level of 2.4 m*M*, q_{gln} increased markedly and q_{gluc} dropped to about the level observed before any changes were made. q_{lac} initially

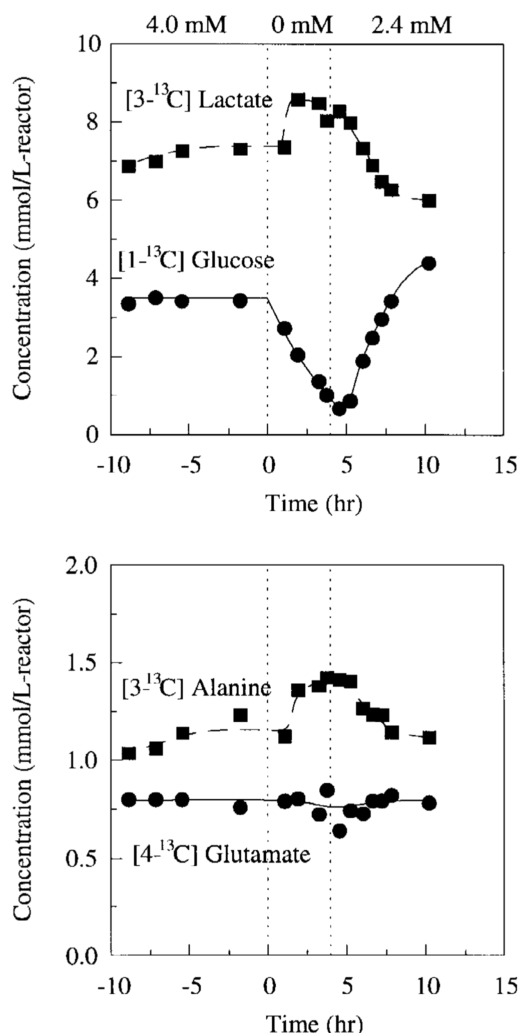


Figure 3. ^{13}C metabolite changes during a brief reduction in glutamine concentration. Metabolite concentrations are all expressed as mmol/L of reactor and feed glutamine concentrations are shown above the graphs. The [4-^{13}C] glutamate concentration includes a contribution from -CH_2-$^{13}CH_2$-CO- in fatty acyl groups.

dropped sharply but eventually recovered to within 15% of the value observed before the changes. q_{Ab} eventually dropped steadily to 24% below the level observed initially. Both q_{NH_3} and q_{ala} were also significantly lower than they were before any changes were made.

Increasing the glutamine concentration also produced a significant increase in a resonance at 61.3 ppm as indicated by the time series of spectra in Figure 6. This resonance was determined to be associated with a methylene group by DEPT 135 analysis and the chemical shift is consistent with that of [1-^{13}C] fructose-6-phosphate and [6-^{13}C] glucose-6-phosphate. Also shown in the figure is the β-[1-^{13}C] glucose resonance. The increase in the resonance at 61.3 ppm occurred 1.2 h after glutamine was restored to the feed and coincided with the time that the glucose concentration began to increase.

The addition of glutamine also resulted in either partial or complete recovery of the concentrations of cystine, isoleucine, leucine, lysine, phenylalanine, tyrosine, threonine, and valine. The aspartate and asparagine concentrations recovered very slowly and, in both cases, only partially. The synthesis of proline was reduced to well below levels observed before any changes were made. Production of glycine ceased several hours after the glutamine concentration was increased, and consumption of glycine resumed shortly thereafter.

Response to a Prolonged Reduction from 2.4 to 1.2 mM in Feed Glutamine Concentration

The feed glutamine concentration to the system was maintained at 2.4 m*M* for approximately 25 h; at this feed level, the residual glutamine concentration was 0.30 m*M*. Subsequently, the feed level was reduced to 1.2 m*M*, which produced a rapid drop in the residual concentration to 0.08 m*M*. The time courses for changes in ^{13}C-labeled metabolites determined in vivo are shown in Figure 7. The concentrations of extracellular primary metabolites determined off-line and the associated specific rates are summarized in Figure 8. A slight reduction in the rate of glycolysis occurred as evidenced by a gradual increase in the [1-^{13}C] glucose level and a slow drop in the [3-^{13}C] lactate level. The concentration of [3-^{13}C] lactate subsequently increased slightly before the glutamine concentration in the feed was increased. The time course for total glucose determined off-line confirmed the in vivo [1-^{13}C] glucose results. However, for total lactate, no drop in production was observed, suggesting that lactate formation from some source other than glucose was slightly enhanced. No significant change was detected in the level of [3-^{13}C] alanine or total extracellular alanine.

The reduced glutamine concentration also produced an almost immediate 50% reduction in q_{gln}. In addition, q_{NH_3} was reduced, but only by approximately 30%, while q_{O_2} was rapidly reduced by approximately 20%. A slight increase in the concentration of antibody was observed, but to within the error of the calculated rates, no significant change was observed in q_{Ab}.

In response to the reduced glutamine concentration, the level of labeling in the [4-^{13}C] glutamate resonance was slightly reduced. This resonance also includes a contribution from -CH_2-$^{13}CH_2$-CO- groups in fatty acyl chains (Cohen, 1987), and a change in the rate of incorporation of label into these groups could have produced the observed change. However, this is unlikely, because in our previous work we observed that with complete discontinuation of ^{13}C-substrate infusion, labeling in lipids decreased at extremely slow rates (Mancuso et al., 1994) probably due to slow lipid turnover rates.

The time courses for extracellular amino acid concentrations are shown in Figure 9. In general, the changes observed were much slower and smaller than those produced when glutamine was completely omitted from the medium. The concentration of aspartate showed the most significant change, decreasing to below the detectable limit 8 h after the

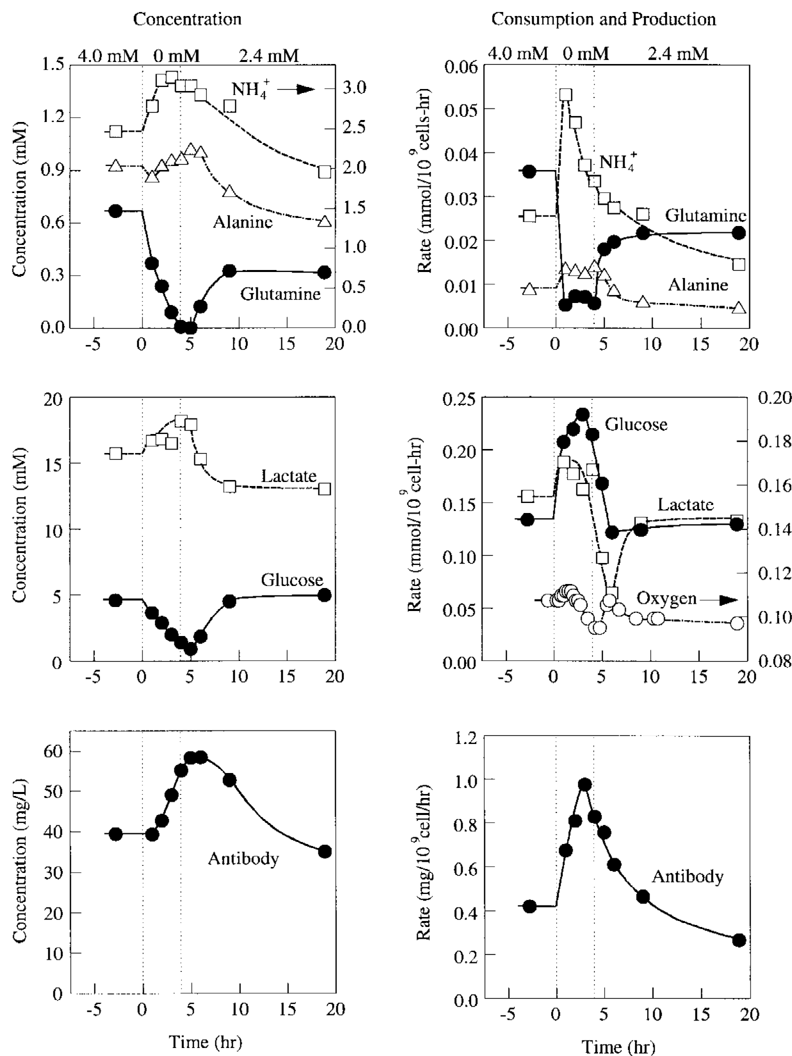


Figure 4. Changes in concentrations and specific rates of extracellular primary metabolites and antibody in response to a brief reduction in glutamine concentration. Concentrations are expressed as mmol/L of recirculating medium, and the feed glutamine concentrations are shown above the graphs.

glutamine feed concentration was reduced. The concentrations of asparagine and glutamate were also reduced. A marked but slow increase in the concentration of lysine, phenylalanine, and glycine was observed; smaller increases were observed for cystine, threonine, and tyrosine.

Incorporation of ^{13}C into lipids was determined by monitoring the increase in the resonance at 30.1 ppm (Fig. 10), which represents -$^{13}CH_2$- groups in the center of saturated regions of fatty acyl chains. The reduction in glutamine concentration did not reduce the rate of increase of this resonance.

Response to an Increase in Feed Glutamine from 1.2 to 4.0 mM

Eleven h after the feed glutamine concentration was reduced to 1.2 m*M*, it was increased to 4.0 m*M;* simultaneously, the recirculating medium was spiked with 5 mL of medium containing 31 m*M* glutamine (equivalent to a 0.84 m*M* increase in the recirculating medium). The level of [1-^{13}C] glucose in both the feed and the spike media was maintained at 17 m*M*. In response to these changes, the residual glutamine concentration abruptly increased to approximately 0.8 m*M* (Fig. 7) which resulted in a rapid three-fold increase in the rate of glutamine consumption and a two-fold increase in the rate of ammonium production (Fig. 8). Small increases were also observed in the rate of oxygen consumption and alanine production. Both off-line analysis and in vivo NMR analysis indicated that the concentration of glucose dropped slightly and the concentration of lactate increased steadily following the change. The intensity of the glutamate-4 resonance at 34.2 ppm increased significantly during that time.

Large increases were observed in the levels of extracellular aspartate, asparagine, and glutamate. More gradual increases were observed in the level of proline, isoleucine, and leucine. Significant reductions were observed in the concentration of lysine and glycine, and very slight reductions were observed for cystine, phenylalanine, and tyro-

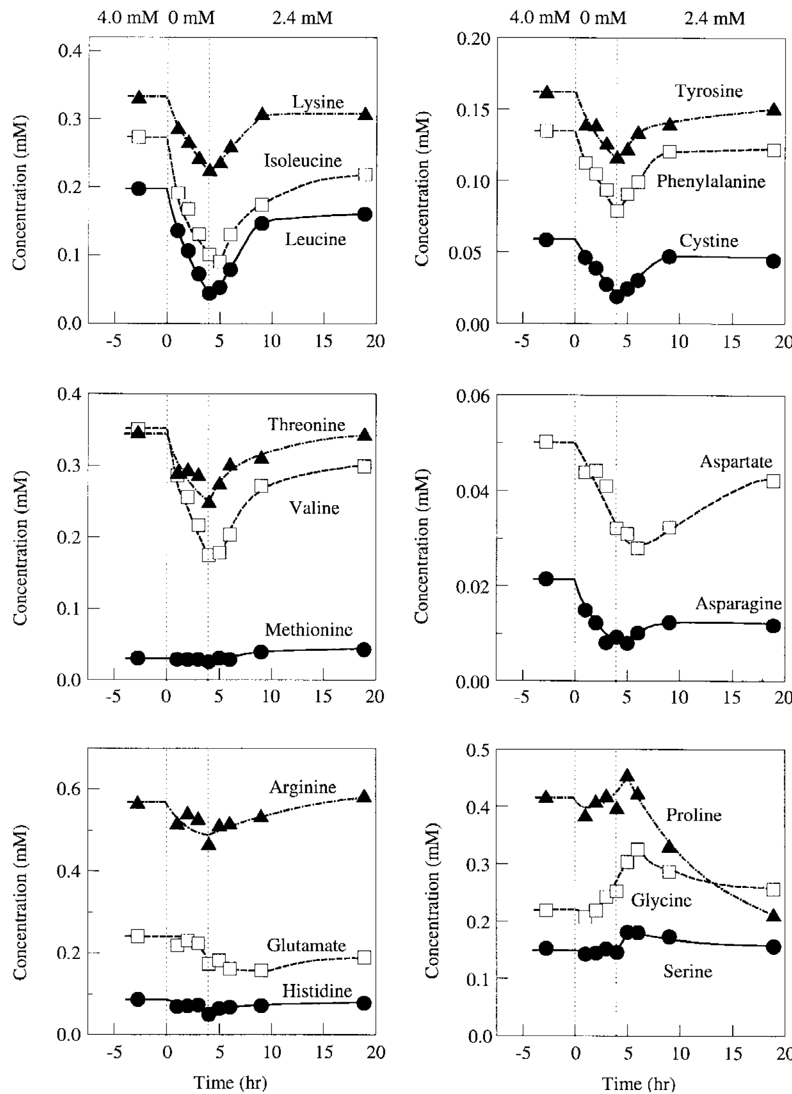


Figure 5. Changes in concentrations of amino acids in response to a brief reduction in glutamine concentration. Concentrations are expressed as mmol/L of recirculating medium, and the feed glutamine concentrations are shown above the graphs.

sine. The rate of incorporation of ^{13}C into -CH_2- of lipids was not affected by the increased glutamine level (Fig. 10). Antibody synthesis was also essentially unaffected by the change (Fig. 8).

DISCUSSION

Altering the extracellular glutamine concentration produced complex changes in primary and secondary metabolism of 4A2 hybridomas. For the brief reduction from 4 to 0 m*M* glutamine, large changes were observed in the consumption of metabolites normally associated with energy production. Nitrogen metabolism and antibody synthesis were also markedly altered. For the prolonged reduction from 2.4 to 1.2 m*M*, much smaller changes were observed, even though the residual concentration of glutamine with a feed concentration of 1.2 m*M* was very low. Each of these effects is discussed in the following sections.

Effect of the Brief Reduction in Glutamine Concentration

Energy Metabolism

With the brief reduction, energy metabolism was perturbed as indicated by the increase in the rate of glycolysis. The consumption rate of glucose and production rate of labeled and total lactate and alanine were all increased. The change in energy metabolism began well before the concentration of residual glutamine had reached 0 m*M;* noticeable changes were detected after the glutamine concentration had dropped to only 0.37 m*M*. The lack of significant change in the [4-^{13}C] glutamate resonance, which reflects the level of labeling in [4-^{13}C] α-ketoglutarate (Chance et al., 1983), suggests that the flux through pyruvate dehydrogenase into the TCA cycle was not affected. The brief reduction also produced an increase in the rate of consumption of other amino acids when the residual glutamine level had dropped

to only 0.37 m*M*. This was especially apparent for energy rich amino acids such as isoleucine, leucine, and lysine.

Theoretical rates of ATP synthesis were estimated with the equation:

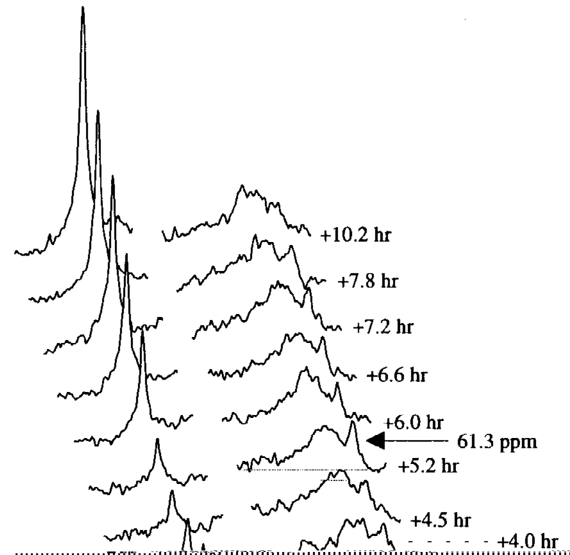
$$q_{ATP} = 5 * q_{O_2} + q_{lac} \quad (3)$$

where q_{ATP} is the specific rate of ATP production. This equation is an approximation that is based on the assumption that the number of moles of ATP produced per mole of atomic oxygen consumed is 2.5 (Stryer, 1995). It neglects ATP produced by substrate-level dephosphorylation of amino acids; however, since the TCA cycle and malate shunt fluxes are so large compared to the dephosphorylation flux, it is a reasonable approximation.

Estimated ATP synthesis rates during the brief reduction in glutamine concentration are shown at the top of Figure 11. The rate initially increased slightly from 0.69 mmol/10^9 cells-h, due to the increased rate of lactate formation. The ^{13}C data shown in Figure 3 indicate that glucose was the primary source of the additional lactate. Within 3 h of the glutamine reduction, q_{ATP} slowed and eventually declined by 17%. This resulted from the decrease in the rate of oxygen consumption, which occurred in spite of the increase in consumption of many amino acids. Restoration of glutamine to the feed resulted in an apparent increase in energy production from glutamine and reversed many of the changes. The q_{ATP} level recovered to within 5% of its original value, 24 h after glutamine was restored to the feed.

Due to limitations in the accuracy of the OUR measurements, the error in the calculated ATP rates is probably in the range of to 10–20%. Thus, the values shown in Figure 11 are only approximate. However, the lack of scatter in the q_{ATP} values suggests that the data are fairly precise, so that statements on the effects of glutamine concentration changes on relative values of q_{ATP} are probably valid.

The cause of the delayed reduction in oxygen consumption, which began approximately 3 h after glutamine was omitted from the feed, is not immediately apparent. It was not likely due to depletion of glycogen, which does not appear to be present at a significant level in 4A2 hybridomas. Glycogen-producing cells cultured on [1-^{13}C] glucose typically display a resonance at 103 ppm (Megnin et al., 1989). Another possibility is that oxygen consumption slowed as the glutamine concentration dropped to below 0.1 m*M,* and the activity level of pyruvate dehydrogenase was not adequate to allow an increase in flux from glucose to



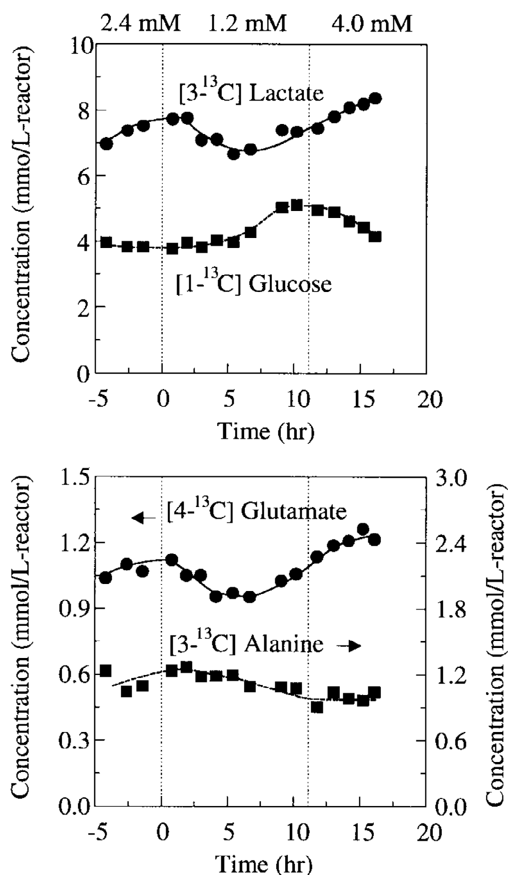


Figure 7. ^{13}C Metabolite changes in response to changes in feed glutamine concentration from 2.4 m*M* to 1.2 m*M* and from 1.2 m*M* to 4.0 m*M*. Metabolite concentrations are all expressed as mmol/L of reactor. The [4-^{13}C] glutamate concentration includes a contribution from -CH_2-$^{13}CH_2$-CO- in fatty acyl groups.

acetyl-CoA to compensate for the reduced TCA cycle flux from glutamine. This possibility is supported by the lack of significant change in the [4-^{13}C] glutamate level as described above.

The sudden increase in the resonance associated with either, or both, [1-^{13}C] fructose-6-phosphate and [6-^{13}C] glucose-6-phosphate that coincided with the increase in [1-^{13}C] glucose concentration (following the re-incorporation of glutamine in the medium) may indicate that some enzyme downstream of hexokinase is important in regulating the glycolytic flux (Lowry et al., 1964). The exact enzyme involved cannot be determined from the limited data acquired. Nevertheless, this observation demonstrates the potential of NMR for examining regulatory phenomena in key pathways in vivo. Such phenomena could be studied in far greater detail with higher field spectrometers with better field homogeneity characteristics (e.g., Gillies et al., 1994) than the spectrometer used in this work.

Nitrogen Metabolism

The rate of assimilation of amino groups was significantly reduced as indicated by the abrupt increase in the ammonium level immediately following the reduction in glutamine concentration (Fig. 4). This change rapidly reversed without additional changes to the culture environment and within 4 h, the ammonium concentration approximately normalized. The ammonium release does not appear to be the result of an inadequate supply of energy, based on the calculated ATP synthesis rates (Fig. 11), but rather a disruption of anabolism triggered by the rapid decline in glutamine concentration.

Antibody Synthesis

A very significant finding of this study was that during the reduction in extracellular glutamine from 0.67 to ~0 m*M*, the specific production rate of antibody more than doubled. Previously, we reported a similar large increase in antibody synthesis with a 1.2 to 0.3 m*M* reduction in extracellular glutamine concentration (Sharfstein et al., 1994). The increased rate of production probably does not represent leakage of intracellular antibody, because that concentration in hybridomas is very low (Reddy et al., 1992) and the extracellular concentration increased very gradually over a timespan of 4 h.

Effect of the Prolonged Reduction and Restoration of Glutamine Concentration

Energy Metabolism

Reducing the feed glutamine concentration from approximately 2.4 to 1.2 m*M* (0.30 to 0.08 m*M* residual) had a significant effect on primary metabolism as indicated by the change in the rate of oxygen consumption. The ^{13}C data indicate that the rate of glycolysis did not increase (in fact a slight reduction occurred). No decrease in total lactate production was observed (Fig. 8), which suggests that a small amount of additional lactate was produced from substrates other than glucose. The data in Figure 4 indicate that glucose consumption rates as high as 0.24 mmol/10^9 cells-h are possible for this cell line. Thus, the lack of increase in glycolysis observed with the large reduction in glutamine consumption indicates that metabolism was not energetically limited by glutamine. With the drop in oxygen consumption, the theoretical ATP synthesis rate dropped by almost 20% (Fig. 11). These results are similar to those we observed previously for 4A2 cells grown in a hollow fiber bioreactor at higher glutamine concentrations. A reduction of feed glutamine from 4.0 to 1.7 m*M* (1.2 to 0.3 m*M* residual) produced a large change in the rate of glutamine consumption, but little change in glucose consumption and lactate production (Sharfstein et al., 1994). These results are also consistent with the observations made by Miller et al. (1989) for a 0.2 to 3.0 m*M* step change (as described in the Introduction), in that increased glutaminolysis did not have an immediate effect on glucose consumption.

A lack of response in glucose catabolism with large changes in glutamine catabolism suggests that the high rates

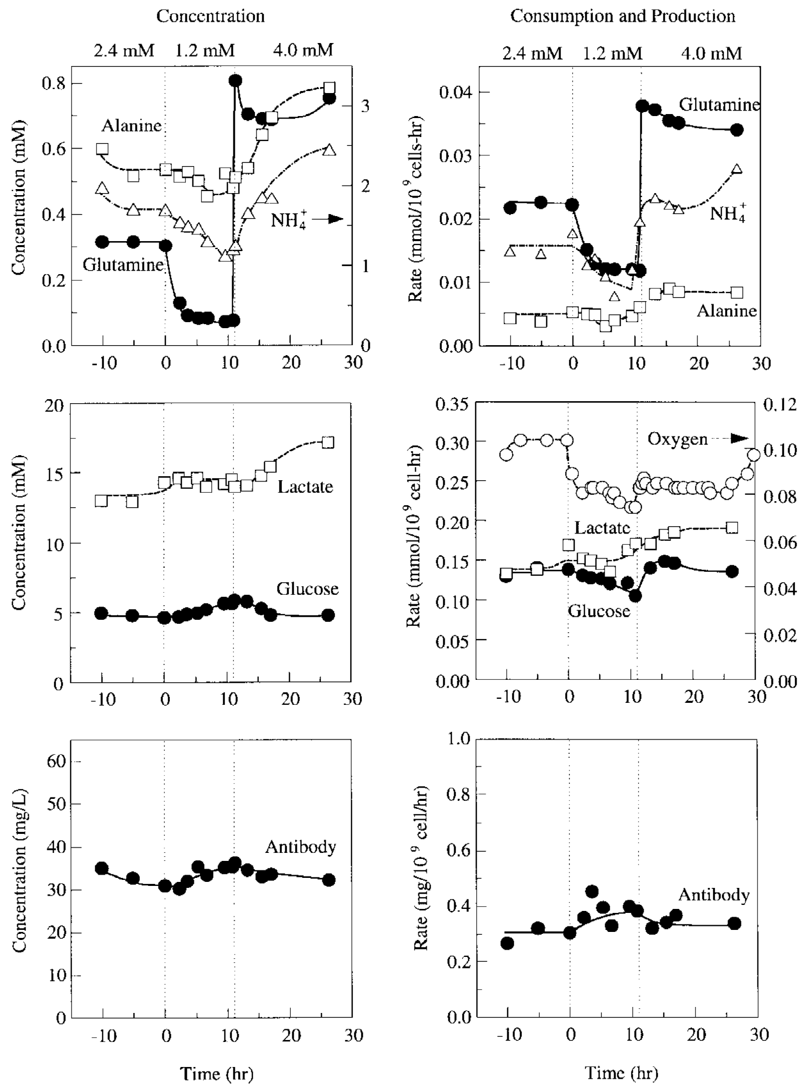
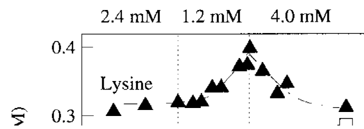
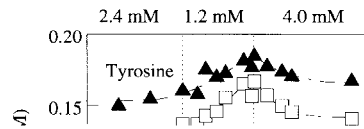


Figure 8. Changes in concentrations and specific rates of extracellular primary metabolites and antibody in response to changes in feed glutamine concentration from 2.4 m*M* to 1.2 m*M* and from 1.2 to 4.0 m*M*. Concentrations are expressed as mmol/L of recirculating medium and the feed glutamine concentrations are shown above the graphs.

of glucose and glutamine consumption observed for hybridomas are important for something other than energy production. Newsholme has proposed that lymphocytes and transformed cells consume more glucose and glutamine than is necessary to meet cellular energy needs to allow accurate regulation of fluxes entering secondary pathways from primary pathways (Newsholme et al., 1985; 1991). Excess energy produced by such high fluxes is dissipated with futile cycling, which also plays an important role in regulation of primary pathways (Newsholme et al., 1992). Glycolytic futile cycles have been observed to occur at higher rates in tumor cells than in non-tumor cells in studies with deuterium-labeled glucose in rats (Torosian et al., 1993). Calorimetric studies have demonstrated that futile cycling occurs in both neoplastic and non-neoplastic cells grown in culture (Gnaiger and Kemp, 1990). The futile cycles that contribute to the additional heat that is dissipated may occur in the glycolytic and glutaminolytic pathways. A futile cycle associated with oxidative phosphorylation may also be important for hybridomas, because uncoupling of mitochondrial proton influx from membrane-bound ATPase activity can be a significant means of dissipating energy that would normally be produced from reduced nucleotides (Brand et al., 1994).

The results for the prolonged reduction contrast sharply with those for the brief reduction. The increased rate of glycolysis observed when the glutamine concentration had declined to only 0.37 m*M* during the brief reduction indicates that some parameter other than extracellular glutamine concentration is important in regulating the glycolytic flux during abrupt changes. One such possible parameter is the magnitude of the initial change in q_{gln}. In Table II, the magnitude of the initial reductions in q_{gln} for the experiments described in this study and for a previous study (Sharfstein et al., 1994) are compared. The data indicate that the largest percentage change in q_{gln} was associ-





ated with the reduction from 4 to 0 m*M* glutamine. Thus, the increased consumption rate of glucose and amino acids observed during that reduction could be the result of limitations in the rate of response of intracellular regulatory mechanisms.

For the change from 2.4 m*M* to 1.2 m*M* glutamine, the concentration of [4-^{13}C] glutamate in the reactor [predominately intracellular (Mancuso et al., 1994)] declined by 13%. Thus, the amount of glutamate produced from glucose via pyruvate dehydrogenase declined slightly in response to the reduction of glutamine in the feed. Less flux of glucose into the TCA cycle with decreased glutamine consumption is inconsistent with energetically limited metabolism, which should have resulted in an increase in the concentration of [4-^{13}C] glutamate. Reduced [4-^{13}C] glutamate could have been the result of unlabeled material entering the TCA cycle via pyruvate dehydrogenase. A possible source for such material would be amino acids from proteins catabolized in response to the reduced availability of amino groups from glutamine, which initially enter the TCA cycle at oxaloacetate and then are converted to pyruvate by the malate shunt (Newsholme and Leach, 1983).

In general, the reduced consumption of lysine, tyrosine and phenylalanine, and other changes observed while the residual glutamine concentration was maintained at 0.08 m*M* was very slight. Of the metabolites examined, only the concentration of asparagine and aspartate changed markedly relative to pre-glutamine-reduction levels. Both are present in the medium at a level of only 0.05 m*M* and accordingly, cannot be a major source of either carbon or nitrogen. At normal glutamine levels, approximately half of the asparagine is consumed, whereas aspartate is normally consumed minimally. The carbon skeletons of both enter the TCA cycle as oxaloacetate. Oxaloacetate is important in removing the amino groups from glutamine-derived glutamate by transamination; the amino group is subsequently used for biosynthesis of purines and pyrimidines (McKeehan, 1986). The increased consumption of asparagine and aspartate may

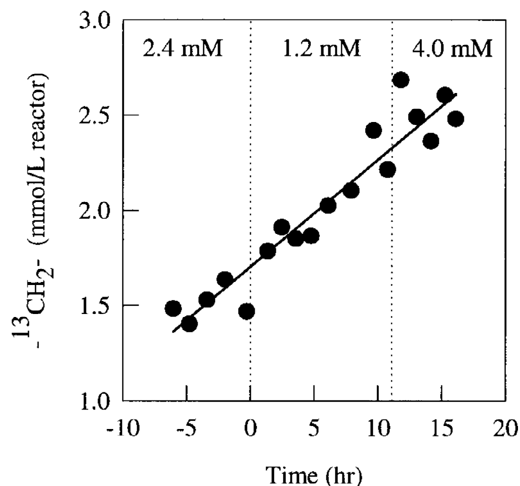


Figure 10. Time course for -$^{13}CH_2$- resonance at 30.1 ppm. These results were determined from spectra processed with 20 Hz exponential line broadening. The feed glutamine concentrations are shown at the top of the graph.

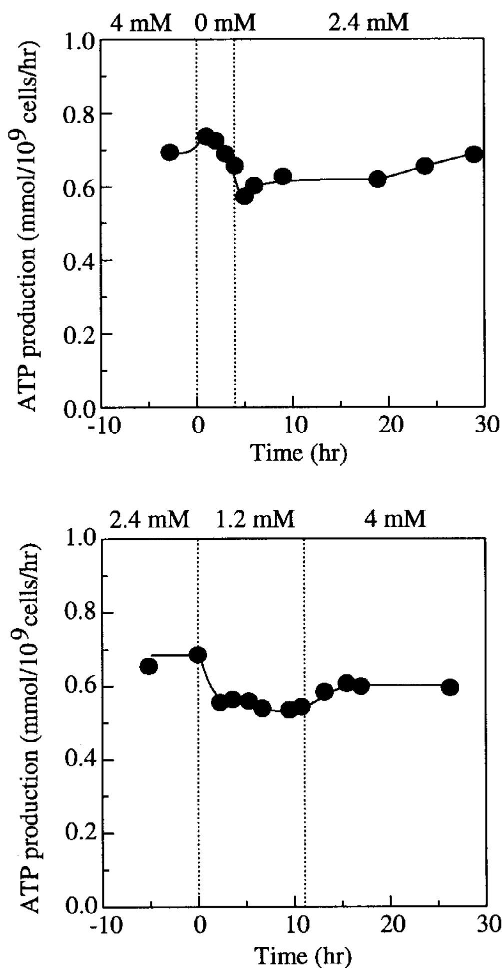


Figure 11. Estimated ATP synthesis rates during the 4 h reduction in feed glutamine concentration from 4.0 to 0 m*M* and subsequent recovery, and the 11 h reduction from 2.4 to 1.2 m*M* with recovery to 4.0 m*M*.

Table II. Changes in glutamine consumption flux following reductions in extracellular glutamine concentration. The data for the first two columns are for this work.

	Experiment (change in feed glutamine concentration)		
	4.0 to 0 m*M*	2.4 to 1.2 m*M*	4 to 1.7 m*M*[a]
Initial flux (mmol/10^9 cells-h)	0.036	0.022	0.035
Flux immediately after reduction (mmol/10^9 cells-h)	0.005	0.015	0.015
Change in flux	86%	32%	57%

[a]From Sharfstein et al. (1994).

indicate that the synthesis of purines and pyrimidines is rate limiting at 0.08 m*M* glutamine.

Lipid Synthesis

The lack of change in the rate of appearance of label in fatty acyl -CH_2- groups (Fig. 10) indicates that neither the level of environmental glutamine nor the glutamine consumption flux affects the rate of lipid synthesis. To compare this result with previously reported work done with this cell line (Sharfstein et al., 1994), the accumulation of -$^{13}CH_2$- in the center of fatty acyl groups was examined retrospectively for a change in feed glutamine concentration from 4.0 to 1.7 m*M* (1.2 to 0.3 m*M* residual, see Fig. 12). The data indicate that the large reduction in glutamine concentration did not affect the rate of incorporation of -$^{13}CH_2$- into fatty acyl groups, which is consistent with the results reported in the current study.

In a previously described model of 4A2 cell metabolism, an NADH balance was used to try to estimate the flux of acetyl-CoA into lipids (Sharfstein et al., 1994). The model predicted that much of the flux of 4-carbon units from glu-

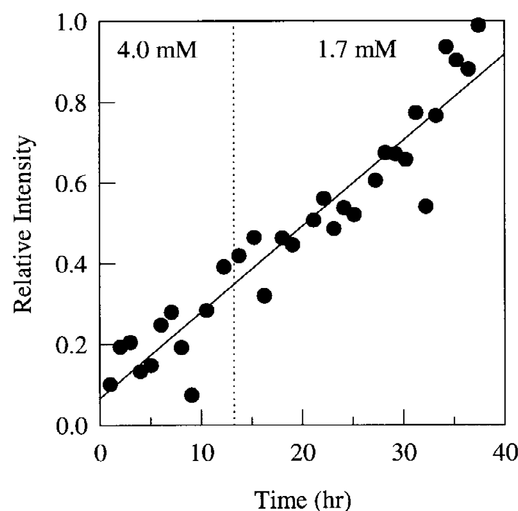


Figure 12. Time course for -$^{13}CH_2$- resonance at 30.1 ppm during a reduction in feed glutamine concentration from 4.0 to 1.7 m*M* for a previously described study (Sharfstein et al., 1994).

tamine was converted to oxaloacetate, to be used in the malate/pyruvate shuttle to transport acetyl-CoA groups (in the form of citrate) from the mitochondrion to the cytoplasm for subsequent lipid synthesis. The estimated rate of acetyl-CoA to lipids from the model was 0.045 mmol/10^9 cells-h (Sharfstein et al., 1994), and was much higher than the rate determined from a time series of NMR spectra of the lipid resonances, 0.007 mmol/10^9 cells-h (Mancuso et al., 1994). If much of the 4-carbon unit flux was used for acetyl-CoA transport, then the rate of incorporation of -$^{13}CH_2$- into fatty acyl groups would be very sensitive to the rate of glutamine consumption (Sharfstein et al., 1994). Because this was not observed experimentally for this study or the study analyzed retrospectively, the results further support the experimentally determined finding that the acetyl-CoA flux to lipids is small, relative to the glutamine consumption flux. The rate determined with the model is probably erroneously high due to the difficulty associated with accurately determining the rate of NADH synthesis.

Antibody Synthesis

Unlike the brief reduction, no significant change in antibody synthesis rate was observed when the feed glutamine concentration was reduced from 2.4 m*M* to 1.2 m*M* (0.3 to 0.08 m*M* residual). For the brief reduction, the increase in q_{Ab} was first observed when the glutamine concentration dropped to 0.24 m*M*. For the reduction from 4.0 to 1.7 m*M* reported previously, q_{Ab} increased at 0.76 m*M* glutamine (Sharfstein et al., 1994). These findings indicate that although stimulation of antibody synthesis can be triggered by a reduction in extracellular glutamine concentration, it is not always triggered at the same concentration of glutamine. It also does not appear to be associated with a single ammonium concentration or change in ammonium concentration, because for the 4.0 to 1.7 m*M* glutamine reduction, the ammonium concentration was approximately 2.3 m*M* and constant, while for the 4 to ~0 m*M* glutamine reduction, the ammonium concentration was 2.8 m*M* and rising (Fig. 4). The mechanism underlying the enhancement in antibody synthesis remains undetermined.

Restoration of Glutamine to the Feed at a Level of 4 mM

Restoration of glutamine to the feed resulted in a large increase in the rate of glutamine consumption and a slight increase in the rate of glycolysis, as indicated by both the ^{13}C and off-line analyses. The marked increase in production of glutamate, proline, and ammonia, as well as the production of aspartate (which is normally consumed at a low rate) indicates that a shift to less oxidative metabolism occurred. This pattern of metabolism is atypical for 4A2 hybridomas, but is similar to that commonly observed in proliferating lymphocytes, where glutamine is used very inefficiently (Newsholme et al., 1985). It suggests that the increased consumption of glutamine was used to provide additional amino groups for secondary pathways, rather than for additional energy production, which is probably a transient over-compensation for the lack of available amino nitrogen for the previous 25 h.

CONCLUSION

The ^{13}C and total metabolite data demonstrate that changes in extracellular glutamine concentration can have several effects on primary and secondary metabolism, depending on the rate at which the concentration is changed. With an abrupt reduction, the flux of energy producing pathways and antibody synthesis can be stimulated, while with more gradual changes, glycolysis and antibody synthesis can remain relatively unaffected. Energy metabolism does not appear to be limited by residual concentrations of glutamine as low as 0.08 m*M,* which suggests that significant futile cycling may be present in the glycolytic and/or glutaminolytic pathways. However, this concentration of extracellular glutamine may be rate limiting for some anabolic pathways, which require glutamine as a source of amino nitrogen.

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