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Alteration of mammalian cell metabolism by dynamic nutrient feeding

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Abstract

The metabolism of hybridoma cells was controlled to reduce metabolic formation in fed-batch cultures by dynamically feeding a salt-free nutrient concentrate. For this purpose, on-line oxygen uptake rate (OUR) measurement was used to estimate the metabolic demand of hybridoma cells and to determine the feeding rate of a concentrated solution of salt-free DMEM/F12 medium supplemented with other medium components. The ratios among glucose, glutamine and other medium components in the feeding nutrient concentrate were adjusted stoichiometrically to provide balanced nutrient conditions for cell growth. Through on-line control of the feeding rate of the nutrient concentrate, both glucose and glutamine concentrations were maintained at low levels of 0.5 and 0.2 mM respectively during the growth stage. The concentrations of the other essential amino acids were also maintained without large fluctuations. The cell metabolism was altered from that observed in batch cultures resulting in a significant reduction of lactate, ammonia and alanine production. Compared to a previously reported fed-batch culture in which only glucose was maintained at a low level and only a reduced lactate production was observed, this culture has also reduced the production of other metabolites, such as ammonium and alanine. As a result, a high viable cell concentration of more than 1.0×10^7 cells/mL was achieved and sustained over an extended period. The results demonstrate an efficient nutrient feeding strategy for controlling cell metabolism to achieve and sustain a high viable cell concentration in fed-batch mammalian cell cultures in order to enhance the productivity.

Introduction

In harnessing the enormous biosynthetic capability of microorganisms, one of the major contributions of process engineering is the manipulation and exploitation of the microorganisms' physiological states. Classic examples include relieving the catabolite repression of antibiotic synthesis by controlled feeding of glucose (Hu and Demain, 1979), and the reduction of ethanol fermentation during baker's yeast production by on-line computer-aided feeding (Wang *et al.*, 1977). More recently the production of undesirable by-products during phenylalanine fermentation was decreased through the employment of an expert system (Konstantinov *et al.*, 1991). One end result of manipulating the physiological states is the redistribution of resources and redirection of metabolite fluxes away from waste product formation and instead toward cell mass or some other desired product(s).

Most of the success stories on enhanced productivity through manipulation of cellular metabolism have been on microbial fermentation systems. However, the potential of exploiting various metabolic states of mammalian cells has also been explored by many since the early stage of modern cell culture processing. Early work focused on the reduction of ammonia formation by controlling glutamine concentration (Glacken *et al.*, 1986). Attempts were also made to alter energy metabolism by controlling glucose concentration (Hu *et al.*, 1987; Hu and Oberg, 1990) or substituting glucose with a more slowly-utilized substrate (Fleischaker, 1982). The results indicate the potential

of manipulating cellular metabolism via on-line nutrient control. These results were consistent with earlier observations by Zielke *et al* (1978). The manipulation of cell metabolism was also achieved through genetic manipulation of cells to allow them to synthesize glutamine from glutamate thus eliminating the need of exogenous glutamine (Bebbington *et al*., 1992). Interestingly these cells often still produce ammonia, albeit to a lesser extent and especially at a low viability (Bibila *et al*., 1994).

The results to date have demonstrated the potential benefits of manipulating energy metabolism of mammalian cells in culture. The reduced metabolite accumulation can possibly lead to a higher viable cell concentration and longer duration of production period. Manipulating energy metabolism has now expanded beyond simply controlling glucose and glutamine concentrations. Through metabolic flux analysis and linear optimization, the contributions of various amino acids to cellular metabolism and their interactions with other nutrients have been examined (Savinell and Palsson, 1992). Manipulation of the amino acid supply rate at stoichiometric ratios has been reported (Xie and Wang, 1994). In that study, altering cellular metabolism was not an original objective, but the investigators unwittingly reduced the conversion of glucose to lactate towards the end of cell cultivation. The cause of this change in metabolism is still not clear. In another study, attempts were made to reduce the conversion of glucose to lactate through on-line control of nutrient feeding by manipulating the glucose concentration at a low level (Zhou *et al*., 1995). The investigators succeeded to reduce lactate production to almost zero near the end of the cultivation. However, the contention that a low level of glucose was the cause of reduced lactate production was not completely supported by the results: although glucose level was high at the end of the culture, the metabolism did not revert to a high lactate producing state. Nevertheless, a high maximum cell concentration with a high viability was achieved.

The metabolism of glucose and glutamine in mammalian cells are interrelated. Both provide the carbon skeletons for cellular molecules and both are catabolized for energy generation. They are considered as partially substitutable and partially complementary substrates (Hu and Himes, 1989). After demonstrating the alteration of cellular metabolism by controlling the glucose concentration at a low level, we manipulated both glucose and glutamine at low concentrations in the follow up experiments. This paper reports the results of such an experiment.

Materials and methods

Hybridoma cell line and culture medium

A clone of a mouse-mouse hybridoma cell line, MAK, which produces IgG monoclonal antibody was used (Zhou and Hu, 1994a). The batch culture medium is a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with transferrin, 2-mercaptoethanol, ethanolamine, L-ascorbic acid, sodium selenite, putrescine and Pluronic F68. The concentrations of glucose and glutamine in this medium are 17.5 and 4 mM respectively. For the fed-batch experiments, the starting medium used was the same as the batch medium except for the glucose and glutamine concentrations which were reduced to 1.4 mM and 0.3 mM respectively. The feeding solution was a 19.8-fold nutrient concentrate of the salt-free DMEM/F12 with the additives mentioned above. The feeding nutrient concentrate did not contain any inorganic salts to minimize effects of the feeding solution on osmolality increase. The glucose concentration in the feeding concentrate was 330 mM and the the glutamine concentration was 99 mM.

Bioreactor set-up

All experiments were performed in an environmentally controlled room maintained at 37°C using a 750-mL glass bioreactor with a working volume of 500 mL. The experimental set up had been described previously (Zhou *et al*., 1995). The bioreactor was monitored and controlled using an IBM PC-XT equipped with a DT-2805 Data Acquisition Board (Data Translation Inc., Marlborough, MA) and two RS-232 boards. The culture pH, dissolved oxygen (DO) concentration, and the masses of the 1.0 N NaOH solution for pH control and the feeding nutrient concentrate were acquired and recorded automatically. The culture pH was controlled between 7.2 and 7.3. Air, oxygen and nitrogen gases were used to maintain the DO at 40% of air saturation. 5% $C0_2$ was maintained in the gas space. Aeration was achieved through the headspace and silicone rubber tubing. Agitation rate of the impeller with two 45° pitched blades (65 mm in diameter) was kept constant at 60 rpm throughout the entire cultivation.

OUR was measured dynamically every hour using a procedure described previously (Zhou and Hu, 1994a). To measure OUR, the DO was increased to 65% of air saturation and the bioreactor was subsequently flushed with nitrogen to decrease the oxygen level in the medi-

um down to 30%. The time profile of DO between 50 and 30% was used to calculatie OUR. The volumetric oxygen mass transfer coefficient (K_La) was determined prior to inoculation using the same method. The K_La value remained constant throughout the cultivation. The average K_La value was 1.13 l/h with only the headspace aeration and 4.58 l/h with both the headspace and silicone rubber tubing aeration. To convert the measured oxygen partial pressure into oxygen concentration, the Henry's coefficient for the culture medium was assumed to be approximately the same as that for pure water (952.27 atm-L/mole) at 37 ∘C. Cumulative oxygen consumption was calculated by numerical integration of OUR over time.

Exponentially growing MAK cells from a seed culture were used to inoculate the bioreactor at a concentration of 1.5×10^5 cells/mL. The initial glucose and glutamine concentrations were 1.4 and 0.3 mM respectively. OUR was measured and the cumulative oxygen consumption was calculated on-line. Using a previously established stoichiometric ratio between glucose and oxygen from batch cultures, the amount of glucose consumed was calculated from the oxygen consumption. The culture was operated in a batch mode until glucose concentration reached 0.50 mM as estimated by on-line calculation. At that time point feeding of the nutrient concentrate was initiated.

Off-line analysis

During the cultivation period, samples were taken periodically for off-line analysis. Fresh samples were used to determine cell, glucose and lactate concentrations. The cell concentration was estimated by microscopic counting with a hemocytometer. Cell viability was determined by the trypan blue dye exclusion method. The glucose and lactate concentrations were measured enzymatically using a YSI Model 27 analyzer (Yellow Springs Instruments, Ohio). The ammonia concentration was determined using an enzyme-based assay kit (Sigma Chemical Co., St. Louis, MO). The osmolality was measured with a vapor pressure osmometer (Wescor Inc., Logan, UT). The antibody titer was assayed by HPLC using a protein G affinity chromatography column (PerSeptive Biosystems, Cambridge, MA). Amino acids were analyzed by HPLC using reversed-phase chromatography and precolumn derivatization with *ortho*-phthaldialdehyde (OPA) (Zhou *et al*., 1995).

On-line stoichiometric nutrient feeding strategy

The feeding rate was based on the amount of glucose estimated to have been consumed by the cells over a discrete time period (in this case, one hour), as described previously (Zhou *et al*., 1995. To determine the rate at which the nutrient concentrate should be fed, OUR was integrated over time to give the amount of oxygen consumed. The amount of glucose consumed is then estimated by dividing the oxygen consumption with the stoichiometric ratio of oxygen to glucose established from previous experiments. The ratio of oxygen to glucose consumption was periodically updated based on historical data to take into account the shift in cell metabolism.

Results and discussion

Fed-batch culture

In a previously reported fed-batch culture using a 15.5-fold concentrated nutrient mixture, the concentrations of the amino acids in the culture were observed to fluctuate over time (Zhou *et al*., 1995). Seventeen amino acids were measured and the ratios of their cumulative consumption were calculated and compared to the composition of the feeding concentrate. Three amino acids – tyrosine, leucine and methionine – were found to be consumed at a rate faster than that at which they were fed. In this study, the concentrations of these three amino acids in the feed solution were increased to their stoichiometric consumption levels. Furthermore, the ratio of glutamine to glucose (mole glutamine per mole glucose) in the feeding solution was decreased from 0.36 in the previous fed-batch culture (Zhou *et al*., 1995) to 0.30. The rationale was to maintain glutamine at a lower level to reduce ammonia and alanine production.

A high viable cell concentration of about $1.0 \times 1O^7$ cells/mL was obtained and maintained at that level for about 70 h (Figure 1). The IgG concentration reached over 250 mg/L compared to 8 mg/L in a batch culture. The glutamine concentration stayed relatively constant throughout the cultivation period. The glucose concentration was maintained in a narrow range for 115 h before it began to increase. Lactate accumulated to 17 mM and ammonia concentration reached 5 mM. A total of 88 mL of feeding was added to the culture which had an initial volume of 500 mL. The

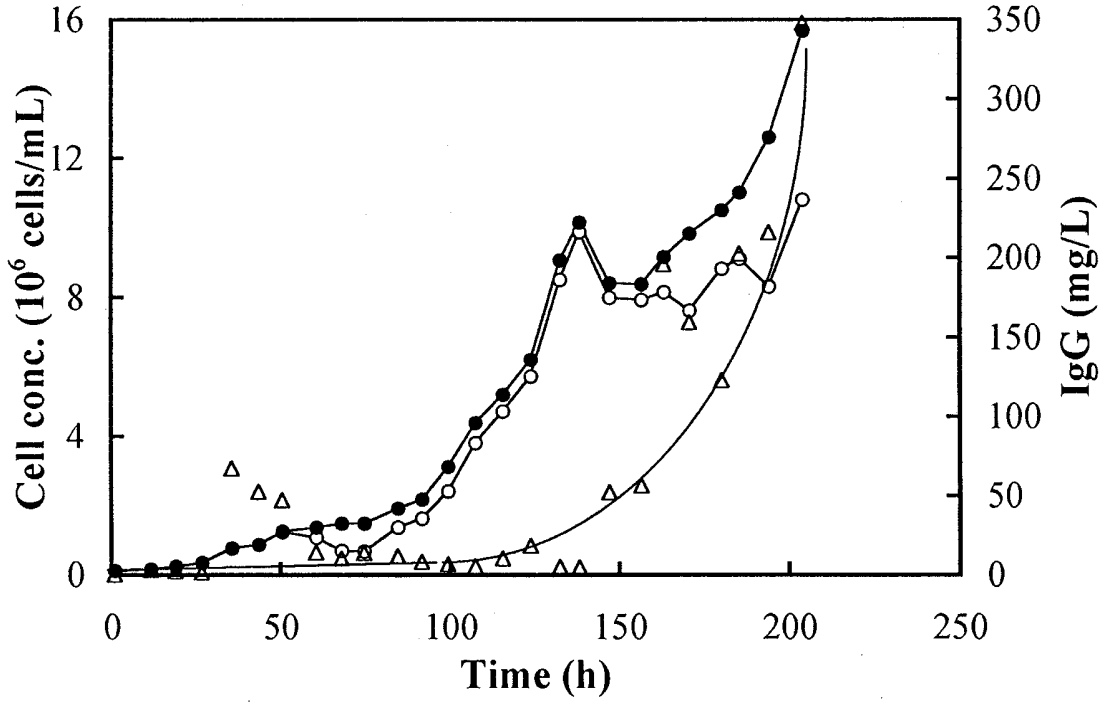

Figure 1a.

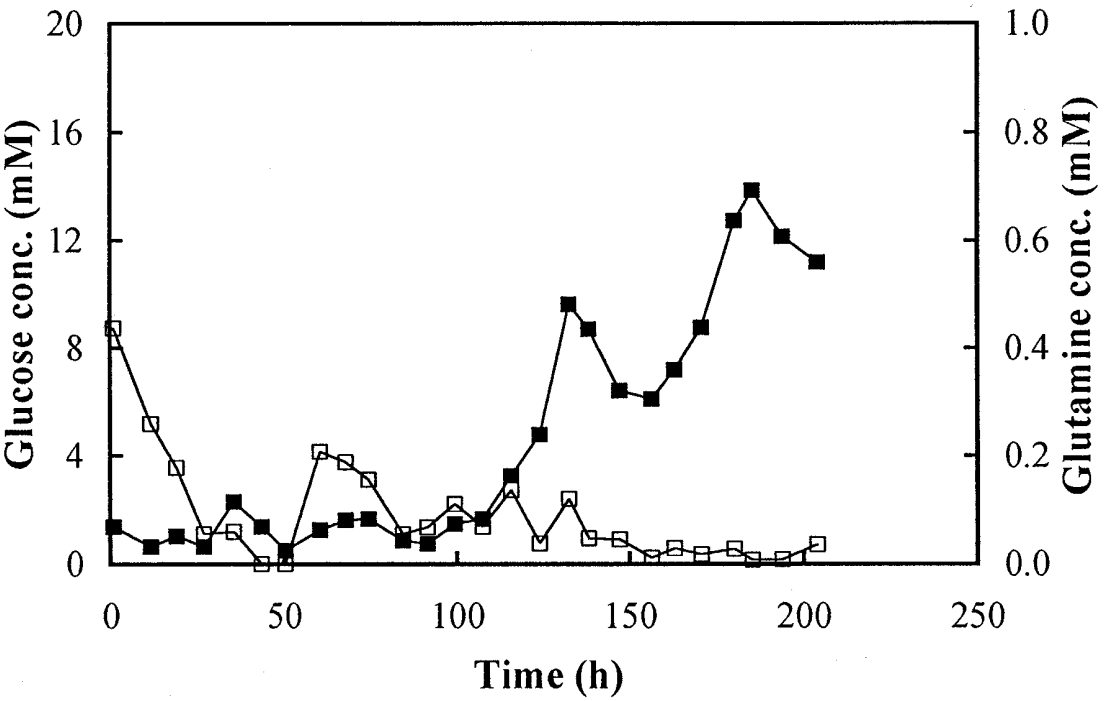

Figure 1b.

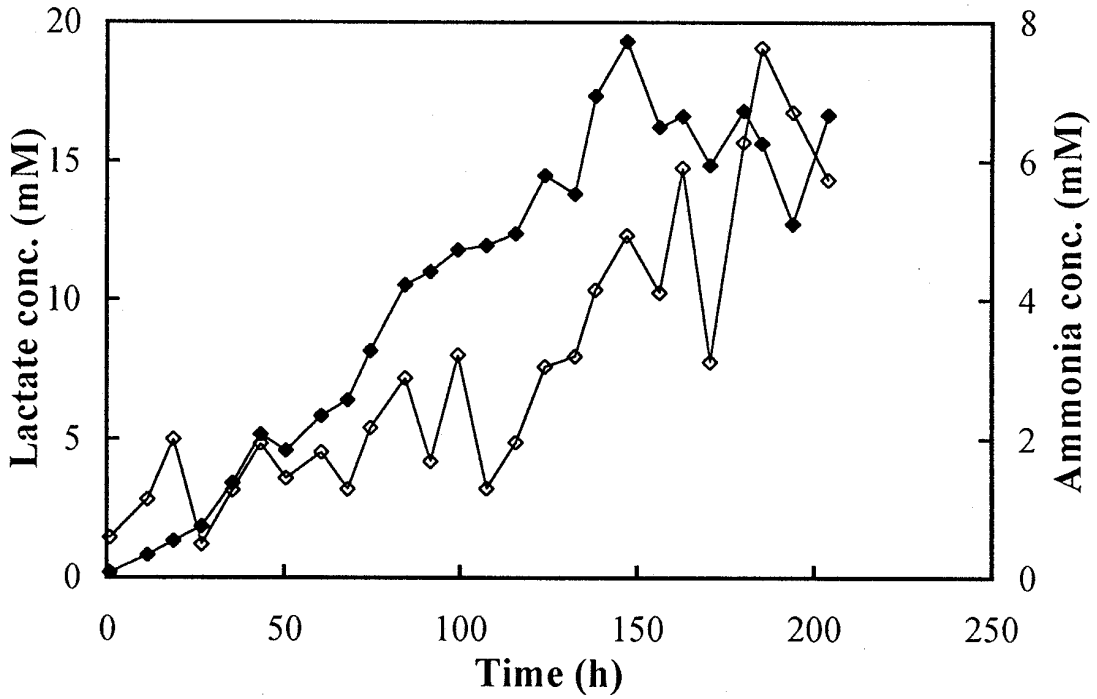

Figure 1a–c. Fed-batch culture kinetics of hybridoma cells in a chemically defined medium. Symbols: concentrations of total cells: (●): viable cells: (○); IgG: (△); glucose: (■); glutamine: (□); lactate: (♦), and ammonia: (◊).

osmolality increased to 400 mosm/kg by the time the culture ended.

The concentrations of all the essential amino acids did not change much from their initial concentrations except for arginine which accumulated in the late culture stage (data not shown). A number of non-essential amino acids often accumulate during batch and fed-batch cultures. In this study, only about 1 mmole/L serine and less than 0.3 mmole/L glycine accumulated in the late culture stage (data not shown). The only non-essential amino acid that accumulated to a significantly high level was alanine which reached 3.7 mM at the end of cultivation.

The cumulative consumptions of glucose, glutamine and oxygen, as well as the cumulative production of lactate and ammonia are shown in Figure 2. The consumption of all three nutrients, as well as the production of lactate, alanine and ammonia continued throughout the cultivation. Lactate acumulation followed glucose consumption closely at the beginning, but remained relatively invariant after 130 h.

Stoichiometric ratios

During the cultivation OUR was measured every hour. From the cumulative oxygen consumption the amount of glucose consumed was estimated and a stoichiometric amount of feeding nutrient solution was added. All other nutrients were added at a fixed ratio to glucose as determined by their stoichiometric ratios to glucose in the feed solution. The initial ratio of glucose consumption to oxygen consumption was 0.44 mmole glucose/mmole oxygen as determined from previous experiments. During the cultivation, however, the ratio changed and had to be adjusted off-line periodically. These adjustments were necessary to avoid the deviation of glucose and glutamine concentrations from the set points of 0.5 and 0.2 mM respectively. In the presentation of the results, the cumulative consumption was normalized to the initial culture volume.

The cumulative consumption of glucose, glutamine and oxygen, and the cumulative production of lactate, ammonia and alanine were plotted against one another to discern their stoichiometric relationships. The stoichiometric ratio between lactate production and glucose consumption was calculated to be initially equal to 0.97 mmole lactate/mmole glucose, but decreased

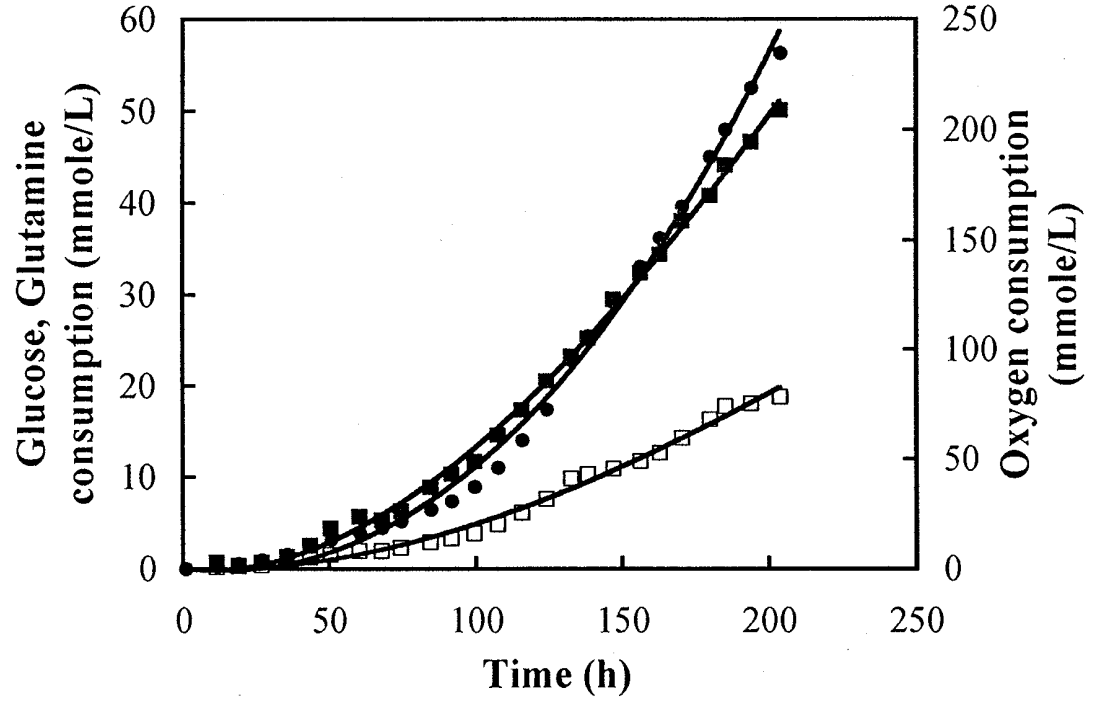

Figure 2a.

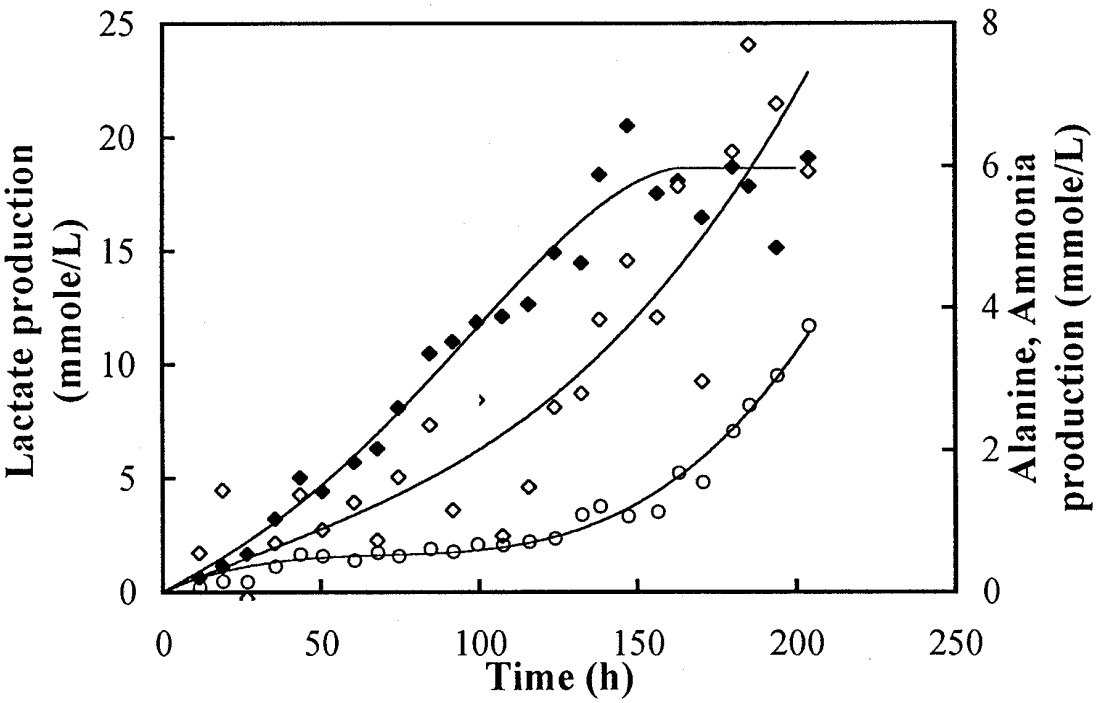

Figure 2a–b. Cumulative nutrient consumption or metabolite production during the fed-batch culture. Symbols: cumulative consumption of glucose: (■); glutamine: (□), and oxygen: (●); and cumulative production of lactate: (♦); ammonia (◊); and alanine: (○).

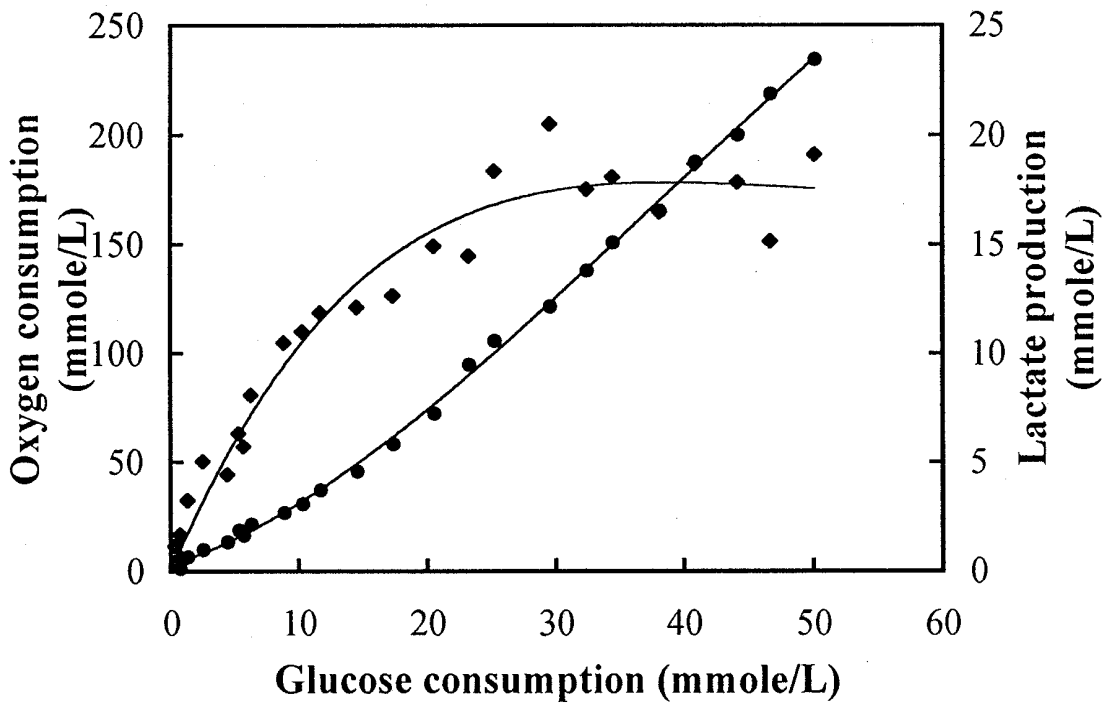

Figure 3. Stoichiometric ratios of oxygen consumption (●) and lactate production (♦) to glucose consumption.

later to a very low value of 0.05 mmole lactate/mmole glucose (Figure 3). This is consistent with the lactate concentration profile between 150 and 210 h, during which almost no lactate was produced although over 20 mmole/L of glucose was consumed. The stoichiometric ratio of oxygen to glucose consumption followed a different trend. Initially, the stoichiometric ratio was lower; the average value for the first fifty hours was 2.2 mmole oxygen consumed/mmole glucose consumed. This value, however, slowly increased and reached 5.0 mmole oxygen/mmole glucose by the end of the culture. The change in these two stoichiometric ratios indicate that the energy metabolism of the cells were predominantly glycolytic at the beginning of the culture and gradually changed to a more oxidative metabolism as the culture progressed.

The relationships of ammonia and alanine production to glutamine consumption are shown in Figure 4. A total of 18 mmole/L of glutamine was consumed and about 3.5 mmole/L of alanine and 7 mmole/L of ammonia were produced. The total amount of the other non-essential amino acids produced during the cultivation was relatively small. Thus, of the 36 mmole/L of nitrogen atoms from glutamine consumption, a net of 10.5 mmole/L resulted in excreted metabolites.

Comparison of different fed-batch cultures

Similar fed-batch cultures have been performed where the glucose and glutamine concentrations were maintained at different levels (Zhou *et al*., 1994b; Zhou *et al*., 1995). The results of two such prior experiments, along with typical batch cultures, were compared to the current one (Table 1). The initial glucose and glutamine concentrations in a batch culture are 17.5 and 4.0 mM respectively. In the first fed-batch culture the initial concentrations were 2.78 mM for glucose and 0.7 mM for glutamine, while those for the second and third fed-batch cultures were 1.40 and 0.3 mM respectively. All the fed-batch cultures were operated in a batch mode until the glucose concentration decreased to the set point at which time the nutrient feeding was initiated. Unlike the experiment presented in this report, in which both glucose and glutamine concentrations were maintained right around their set points, only glucose concentration was manipulated in the first two fed-batch cultures. The glucose concentration was set at 2.5 and 0.55 mM for those two experiments respectively. The glutamine concentration in the culture accumulated from 0.5 to 3 mM in the first culture and remained around 0.5 mM in the second culture as the results

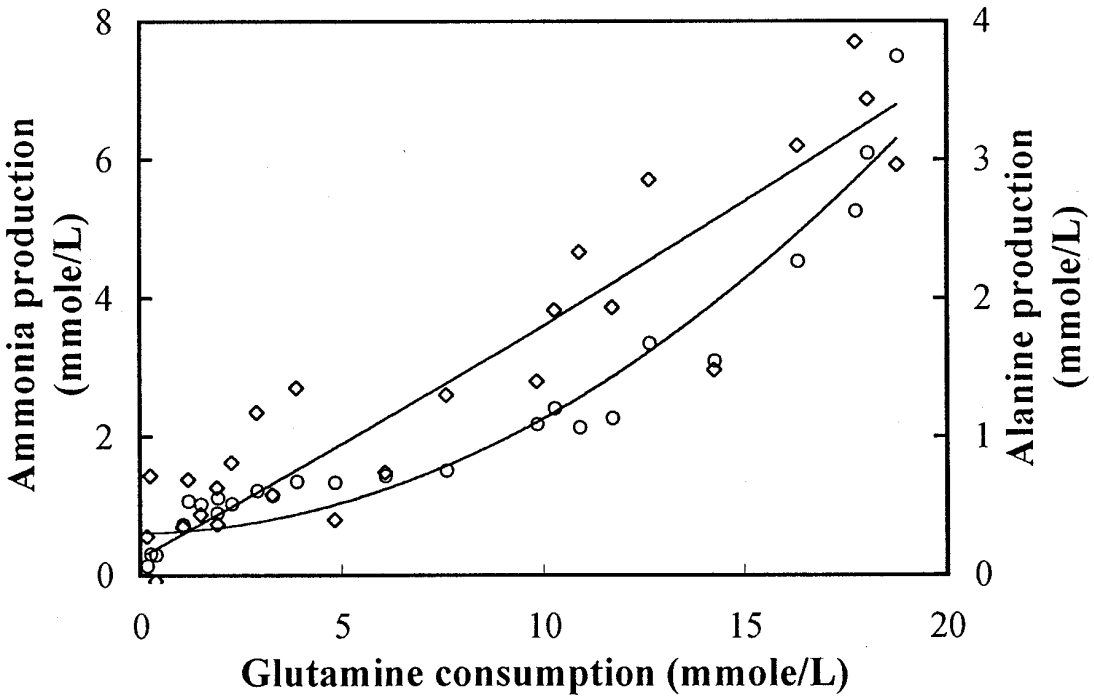

Figure 4. Stoichiometric ratios of ammonia (◊) and alanine (○) production to glutamine consumption.

Table 1. Comparison of results of fed-batch hybridoma cultures with on-line control of nutrient feeding with those of a batch culture

	Batch	Fed-batch 1	Fed-batch 2	Fed-batch 3
Initial glucose conc. (mM)	17.0	2.78	1.4	1.4
Initial glutamine conc.(mM)	4.0	0.7	0.3	0.3
Glucose conc./set point (mM)		2.50	0.55	0.55
Glutamine conc. (mM)	4→0	∼0.5→3.0	∼0.5	0.2 (set point)
Maximal viable cell conc. (10^6 cells/mL)	2.4	7.2	12.0	10.5
Antibody conc. (mg/L)	8	35	60	250
Duration of growth stage (h)	70	100	145	140
Glucose consumption[a] (mmole/L)	12.0	46.0	46.50	27.0
Glutamine consumption[a] (mmole/L)	4.0	15.6	17.0	10.1
Oxygen consumption[a] (mmole/L)	24.0	90.0	143.0	122.0
Lactate production[a] (mmole/L)	18.0	70.0	37.4	17.0
Ammonium production[a] (mmole/L)	3.0	6.5	9.7	4.5
Lactate/Glucose (mmole/mmole)	1.5	1.47	1.60→0.16	0.9→0.05
Oxygen/Glucose (mmole/mmole)	1.85	2.27	1.9→6.0	2.2→5.0
Ammonium/Glutamine (mmole/mmole)	0.75	0.78	0.5	0.31→0.10
Alanine/Glutamine (mmole/mmole)	0.35	0.37	0.34→1.35	0.08→0.42
Osmolality (mosm/kg)	∼350	455	410	400

[a] These data are the cumulative values during the cell growth stage.
→ denotes gradual decrease or increase of these parameters during the cultivation.

of the nutrient feeding control based on the glucose consumption.

The duration of the exponential growth stage was extended significantly in the last two fed-batch cultures. Comparing the first fed-batch culture to the batch culture, the stoichiometric ratios are similar except for the ratio of oxygen consumption to glucose consumption which appears to be slightly higher for the fed-batch culture. However, as pointed out earlier, by manipulating glucose at a lower level, the stoichiometric ratios changed significantly. In the second and third fed-batch cultures, both the lactate-to-glucose and the oxygen-to-glucose ratios changed during cultivation to show a more oxidative mode of metabolism. By further maintaining the glutamine concentration at a lower level in the last fed-batch culture, the accumulation of ammonia and alanine was also reduced. When glutamine was maintained at a concentration greater than 0.5 mM, each mole of glutamine consumed gave rise to 0.5 mole ammonia and 1.35 mole of alanine late in the cultivation. By keeping the glutamine concentration below 0.2 mM those values were reduced to 0.10 and 0.42 for ammonia and alanine respectively.

In the last fed-batch culture a high viable cell concentration was maintained longer. This contributed to the higher final antibody titer compared to the other cultures. Since high levels of ammonia and lactate are presumed to be growth inhibitory, reducing their production is certainly beneficial. Whether the reduced levels of lactate and anunonia in the last fed-batch culture contributed directly to this high viable cell concentration over an extended period is still not known.

The reduced production of lactate was attributed to controlling glucose at a low level. The results from the experiments shown in Table 1 appear to be consistent with this notion. Other fed-batch cultures using the same cell line and with glucose being manipulated at different levels also lent more supporting evidence (unpublished results by M. Hosobuchi in this laboratory). Our results also suggest that the alteration of metabolism is an adaptive, gradual process. The reduction of lactate production occurred over time rather than instantaneously switching from a glycolytic state to an oxidative state. This is consistent with observations reported earlier (Zhou *et al*., 1995). It is in contrast to that observed with a change in oxygen uptake rate upon changing glucose concentration in microcarrier culture of normal diploid epithelial cells (Frame and Hu, 1985). In a previous study with Vero cells, it was observed that lactate production decreased within a few hours after cultivating cells in a low glucose medium (Hu and Oberg, 1990). It should be noted that, in both this and previously reported studies (Zhou *et al*., 1995), at the end of fed-batch cultivation, though the glucose concentration increased beyond the set point, the ratio of lactate produced to glucose consumed was still at a low value. Therefore, under some conditions a high concentration of glucose does not necessarily revert cells to a high lactate production state (at least within the duration of the experiment). It is possible that a low glucose concentration is sufficient for altering cell metabolism to a more oxidative mode (albeit at a slow pace for hybridoma cells), but may not be a necessary condition.

Ammonia is produced mainly from the deamination of glutamine to form glutamate while the transamination reaction of glutamate and pyruvate produces α-ketoglutarate and alanine. The reduced levels of ammonia and alanine production reported in this study are attributed to the lower concentration of glutamine in the culture. Ammonia is also formed from the first order spontaneous degradation of glutamine. lt is thus possible that the reduced ammonia accumulation was the result of reduced spontaneous degradation because of lower glutamine concentration in the culture. However, the reduction of spontaneous degradation of glutamine by reducing its concentration from 0.5 mM in the previous fed-batch culture to 0.2 mM in this fed-batch is very small. A total reduction of 0.33 mmole/L of ammonia accumulation could be directly attributed to decreased spontaneous glutamine degradation assuming a glutamine spontaneous degradation rate constant of $0.0044\ h^{-1}$ (Tritsch and Moore, 1992). This amount is relatively small compared to the total change in the amount of 5 mmole/L of ammonia formed between the two fed-batch cultures. Thus the reduced formation of ammonia in this fed-batch culture is mainly due to a metabolic change that resulted from a reduced concentration of glutamine.

Controlling the concentrations of glucose and glutamine at low levels promotes a change in the metabolism of mammalian cells. In this fed-batch experiment along with previously reported fed-batch cultures it was shown that a change in the levels of glucose and glutamine concentrations has an effect on the amounts of lactate, ammonium, and alanine produced during the culture. Lower levels seem to decrease metabolite production. Whether these changes in the culture enviroment is a direct cause for the longer cell viability and higher cell concentration sustained or a mere effect of the reduced production of the toxic

metabolites is not altogether clear and warrants further investigation.

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