Appl Microbiol Biotechnol (1999) 51: 579–585

ORIGINAL PAPER

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Metabolic responses to different glucose and glutamine levels in baby hamster kidney cell culture

Received: 21 August 1998 / Received revision: 11 November 1998 / Accepted: 17 January 1999

Abstract In this work, a BHK21 clone producing a recombinant antibody/cytokine fusion protein was used to study the dependence of cell metabolism on the glucose and glutamine levels in the culture medium. Results obtained indicate that both glucose and glutamine consumptions show a Michaelis-Menten dependence on glucose and glutamine concentrations respectively. A similar dependence is also observed for lactate and ammonia productions. The estimated value of the Michaelis constant for the dependence of lactate production on glucose $\left(K_{\mathrm{Lac}}^{\mathrm{Glc}}\right)$ was 1.4 ± 0.1 mM and for the dependence of ammonia production on glutamine $\left(K_{\mathrm{Amm}}^{\mathrm{Gln}}\right)$ was 0.25 ± 0.11 mM and 0.10 ± 0.03 mM, at glucose concentrations of 0.28 mM and 5.6 mM respectively. At very low glucose concentrations, the glucose to lactate yield decreased markedly, showing a metabolic shift towards lower lactate production. This metabolic shift was also confirmed by the significant increase in the specific oxygen consumption rate also observed at low glucose concentrations. Although it was highly dependent on glucose concentration, the oxygen consumption also increased with the increase in glutamine concentration. At very low glutamine concentrations, the glutamine to ammonia yield increased, showing a more efficient glutamine metabolism.

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Introduction

Mammalian cells, amongst others baby hamster kidney (BHK) cells, have been widely used in the biotechnological production of vaccines, diagnostics and therapeutics. Nevertheless, partly because of the relatively low cell densities and short production phases, product yields in mammalian cell cultures are typically low, which is undesirable for production purposes. Improvements in mammalian cell production systems are, therefore, likely to be achieved by better medium design and process control. However, these approaches require a greater understanding of the effect of the environment on cell physiology and recombinant protein production.

The metabolism of transformed cells is significantly different from the metabolism of the derived parent cells, glycolysis and glutaminolysis rates often increasing (glucose and glutamine are the main nutrients utilized by mammalian cells) (McKeehan 1982). Glucose is the major carbon and energy source for cell growth, whereas glutamine is the major nitrogen source (Eagle et al. 1958; Levintow et al. 1957; McKeehan 1982, 1986). Owing to their important roles in animal cell growth and their high consumption rates, glucose and glutamine are usually assumed to be the cell-growth-limiting nutrients (Dalili 1990). Glutamine metabolism can provide 30%–65% of the energy required for mammalian cell growth (Reitzer et al. 1979; Zielke et al. 1984). The proportion of each nutrient consumed by the different pathways depends on the metabolic state of the cells, which is also dependent on the nutrient environment.

In mammalian cell culture, waste products secreted from the cells have significant effects on cell growth and metabolite production. Lactate is mainly produced from glucose metabolism, but can also be produced in small amounts from glutamine (Zielke et al. 1980), and ammonium is produced in amino acid metabolism, especially in glutamine metabolism. Several studies on the effects of lactate and ammonium ion on cell growth, metabolism and productivity have been reported (Butler

and Spier 1984; Miller et al. 1988a; Ozturk et al. 1992). Attempts have been made to control cell metabolism and to reduce the production of those metabolites by manipulating the concentration of glucose (Hu et al. 1987), glutamine (Glacken et al. 1986) or both (Ljungreen and Häggström 1994). There is no complete, quantitative description of the glucose and glutamine consumptions (and their interactions) and lactate and ammonia productions, although hybridoma cells have been reasonably well studied (Hu et al. 1987; Ljungreen and Häggström 1994). It is our goal to characterize the physiological behaviour of BHK cells better, through the integrated study of metabolic rates (glucose, glutamine and oxygen consumptions as well as lactate and ammonia productions) as a function of glucose and glutamine concentrations in the culture medium; this integrated work includes the study of the reciprocal regulation of glycolysis and glutaminolysis, of the utmost importance when characterizing physiological behaviour of cells in culture.

Materials and methods

Cell lines

BHK 21A cells, genetically modified to produce a fusion protein consisting of a recombinant human IgG molecule with a cytokine tail, were obtained from Merck KGaA, Darmstadt, Germany.

Cell growth systems

Cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Glasgow, UK) supplemented with 5% fetal calf serum (Sigma, St. Louis, USA). Studies were performed in 25-cm^2 static flasks (Greiner, Frickenhausen, Germany) containing 10 ml medium and 5×10^4 cells ml^{-1} as inoculum. The cells were adapted to the different nutrient conditions of each study (different glucose and glutamine concentrations) by being grown in that medium for at least 48 h. This required medium exchanges every 4 h to avoid nutrient depletion, especially important at the lower nutrient concentrations. These medium exchanges were performed by carefully withdrawing the medium with a pipette and then replacing the same volume of fresh medium.

On the day of the assays, the medium was changed immediately before the beginning of the experiments. During the assays, which lasted 4 h, the cells were incubated with the same medium where they had been growing, but the serum was absent. This was done because of the possible presence of glutamine in the serum, which could have led to increased glutamine concentrations in the culture medium; this was especially important in the assays of low glutamine concentrations. All cultures were performed at 37 °C in a humidified atmosphere containing 7% CO_2.

Cell counting

Cells were counted in a Fuchs-Rosenthal haemacytometer (Brand, Wertheim, Germany). Viable cells were determined by the trypan blue dye-exclusion method. Adherent cells were determined after release from the static surface (Cruz et al. 1997).

Metabolite analysis

All metabolites were quantified enzymatically. Glucose was quantified by the procedure 16-UV (Sigma), glutamine by procedure GLN-2 (Sigma), lactate by UV test number 139084 (Boehringer Mannheim, Mannheim, Germany) and ammonia by UV test number 1112732 (Boehringer Mannheim). All these metabolites were quantified every hour during the 4-h incubation of the cells in the several media containing different glucose and glutamine concentrations. At the end of each assay, the cells were quantified by total protein analysis, using the biuret/bicinchoninic acid reaction (product no. 23225; Pierce, Rockford, USA), to correct any possible difference in the cell concentration present in each experiment.

Oxygen assays

The cells were grown in a similar way to that previously described for the metabolite assays. Before the tests, the cells were removed from the culture surface with the use of trypsin, centrifuged for 10 min at 100 *g* and then resuspended in the different media tested. These homogeneous cell suspensions were then introduced into the electrode chamber. The partial pressure of dissolved oxygen was measured by an oxygen microelectrode (Hansatech, Norfolk, England). The probe was calibrated to 100% dissolved oxygen by sparging air in water and 0% by sparging nitrogen in water. The temperature was kept constant at 37 °C. The oxygen consumption rates were calculated, assuming an air saturation value of 0.21 mM of oxygen at 37 °C (Boraston et al. 1983). The results presented are the average values of at least three experimental assays.

Results

Glucose metabolism

Glucose uptake

The variation of the cell-specific glucose uptake rate with the glucose concentration in the culture medium was studied. The results obtained, presented in Fig. 1, show that glucose uptake rate has Michaelis-Menten behaviour: a high decrease is observed at low glucose concentrations, while at high glucose concentrations the glucose consumption rate tends to a constant value. The maximum glucose consumption rate $\left(q_{\mathrm{Glc}}^{\max}\right)$ was estimated to be 32.5 ± 0.8 nmol $(10^6 \text{ cells})^{-1}$ min^{-1}, while the Michaelis-Menten constant $\left(K_{\mathrm{Glc}}^{\mathrm{Glc}}\right)$ value was esti-

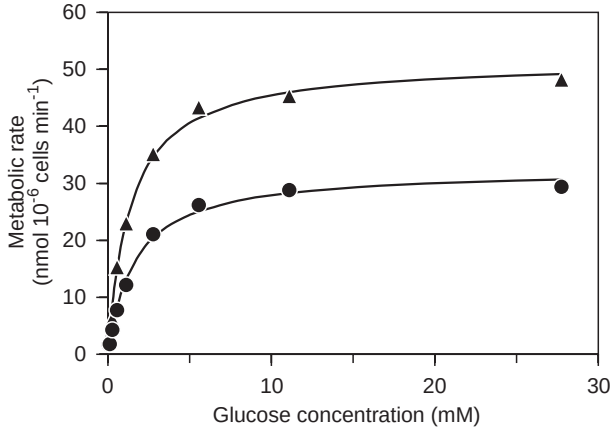

Fig. 1 Cell-specific glucose consumption rate (●) and cell-specific lactate production rate (▲) as a function of glucose concentration. Glutamine concentration was maintained constant at 1.0 mM

mated to be 1.6 ± 0.2 mM glucose {where $q_{Glc} = q_{Glc}^{max}[Glc]/(K_{Glc}^{Glc} + [Glc])$}.

Lactate production

The cell-specific lactate production rate, as a function of glucose concentration, showed similar behaviour to that found for glucose. As can be observed in Fig. 1, Michaelis-Menten behaviour was obtained: lactate production was very low under low glucose concentrations, while at high glucose concentrations lactate production was almost constant. The maximum lactate production rate q_{Lac}^{max} was estimated to be 51.6 ± 1.1 nmol $(10^6 \text{ cells})^{-1}$ min^{-1}, while the Michaelis-Menten constant K_{Lac}^{Glc} value was estimated to be 1.36 ± 0.11 mM glucose.

Glucose to lactate yield

As can be observed in Fig. 2, the glucose to lactate yield is almost constant for the glucose concentrations used. Nevertheless, it decreases dramatically at very low glucose concentrations. This decrease is observed at glucose concentrations below approximately 0.6 mM or 0.1 g l^{-1}.

Glucose uptake as a function of glutamine concentration

As can be observed in Fig. 3, the glucose uptake rate is almost constant under the different glutamine concentrations used. However, a decrease in glucose uptake is observed at very low glutamine concentrations. This is observed for both glucose concentrations used. It is to be noted that, for a certain glutamine concentration, the glucose uptake is higher for the higher glucose concentration, as observed before in the studies on glucose uptake rate as a function of glucose concentration.

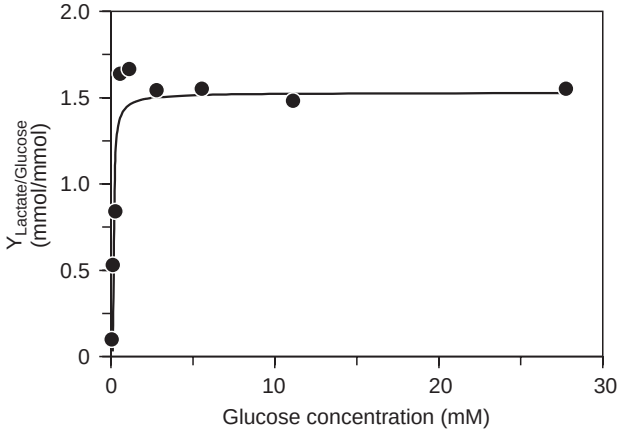

Fig. 2 Glucose to lactate yield (Y) as a function of glucose concentration. Glutamine concentration was maintained constant at 1.0 mM

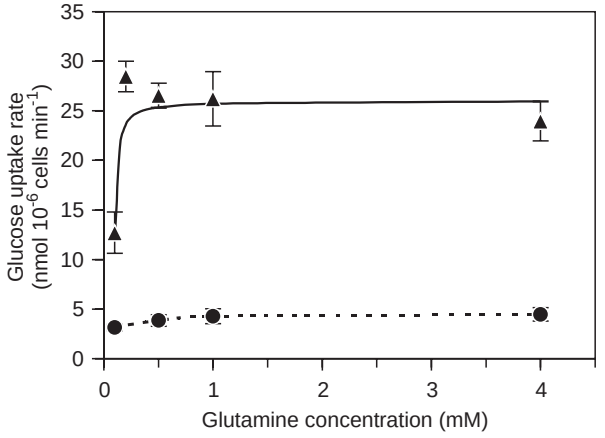

Fig. 3 Cell-specific glucose consumption rate as a function of glutamine concentration, at glucose concentrations of 0.28 mM (●) and 5.6 mM (▲)

Influence of glucose concentration on cell growth

The cell-specific growth rate was studied at different glucose concentrations in the culture medium. The results depicted in Table 1 show that no significant differences were obtained in cell growth rates at glucose concentrations of 0.8, 1.7 and 17 mM.

Glutamine metabolism

Glutamine uptake

The cell-specific glutamine uptake rate as a function of glutamine concentration in the culture medium was studied. The results obtained, presented in Fig. 4A, show that glutamine uptake has Michaelis-Menten behaviour, similar to that already described above for glucose consumption. The maximum glutamine consumption rates q_{Gln}^{max} were estimated to be 6.5 ± 0.6 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} for a glucose concentration of 0.28 mM and 2.00 ± 0.11 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} for a glucose concentration of 5.6 mM. The K_{Gln}^{Gln} values were estimated to be 0.59 ± 0.17 mM for a glucose concentration of 0.28 mM and 0.15 ± 0.04 mM for a glucose concentration of 5.6 mM. It is important to highlight that the glutamine consumption was higher at the lower glucose concentration. This corroborates the complementary nature of these two main nutrients for animal cells.

Table 1 Variation of cell-specific growth rate (μ) with glucose concentration

Glucose concentration (mM)	μ (h^{-1})
0.8	0.021 ± 0.003
1.7	0.022 ± 0.004
17	0.018 ± 0.002

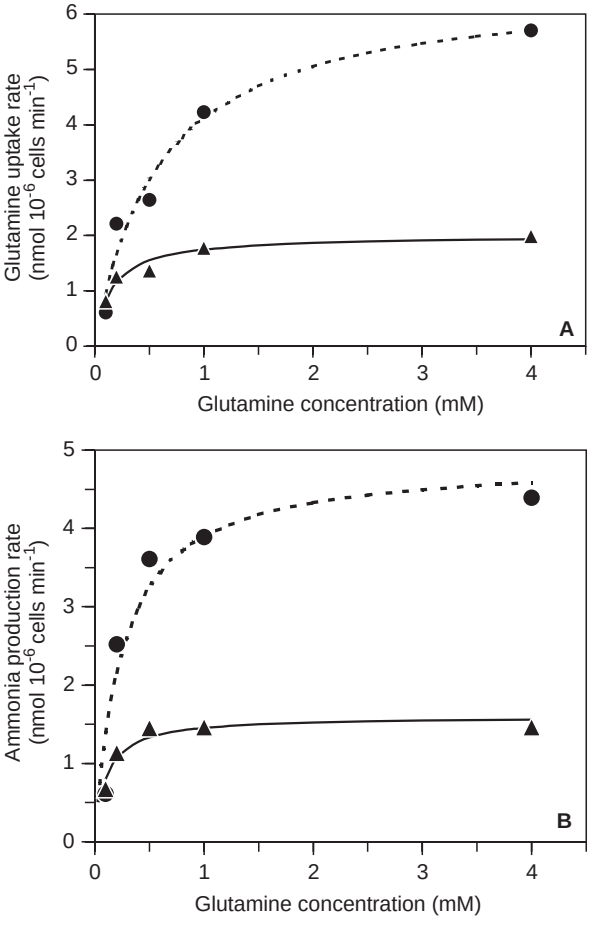

Fig. 4A, B Cell-specific glutamine consumption rate (**A**) and cell-specific ammonia production rate (**B**) as a function of glutamine concentration, at glucose concentrations of 0.28 mM (●) and 5.6 mM (▲)

Ammonia production

The cell-specific ammonia production rate as a function of glutamine concentration in the culture medium is presented in Fig. 4B, similar behaviour to that found for glutamine uptake being obtained. The maximum ammonia production rates q_{Amm}^{max} were estimated to be 4.9 ± 0.6 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} for a glucose concentration of 0.28 mM and 1.60 ± 0.10 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} for a glucose concentration of 5.6 mM. The K_{Amm}^{Gln} values were estimated to be 0.25 ± 0.11 mM for a glucose concentration of 0.28 mM and 0.10 ± 0.03 mM for a glucose concentration of 5.6 mM. All these values are consistent with those obtained in the glutamine uptake assay, including the fact that the ammonia production rate was higher at the lower glucose concentration.

Glutamine to ammonia yield

The glutamine to ammonia yield as a function of the glutamine concentration in the culture medium is presented in Fig. 5. The glutamine to ammonia yield is almost constant for glutamine concentrations above 1 mM, ranging from 0.7 to 0.9 mmol ammonia/mmol glutamine. However, with glutamine concentrations under approximately 1 mM, the glutamine to ammonia yield increases significantly, particularly for the lower glucose concentration (0.28 mM).

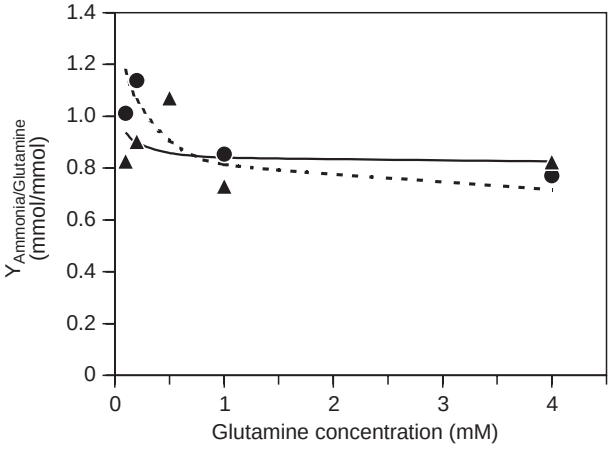

Fig. 5 Glutamine to ammonia yield as a function of glutamine concentration, at glucose concentrations of 0.28 mM (●) and 5.6 mM (▲)

Oxygen consumption

The effects of glucose and glutamine concentrations on the cell-specific oxygen consumption rate (SOCR) were studied at different glucose concentrations, and under two different glutamine concentrations, in order to obtain additional experimental evidence for the observed metabolic shifts. As can be observed in Fig. 6, there is a clear decrease in SOCR with the increase in the glucose concentration. Two phases can be identified in the curves obtained: one at glucose concentrations higher than ap-

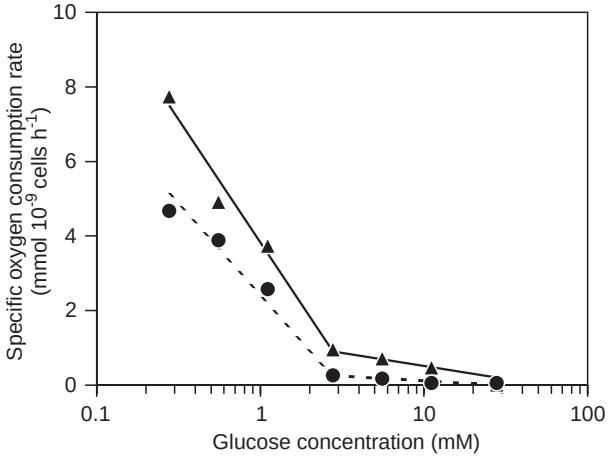

Fig. 6 Specific oxygen consumption rate as a function of glucose concentration, at glutamine concentrations of 0.1 mM (●) and 1.0 mM (▲)

proximately 2 mM, where both the SOCR and its dependence on the glucose concentration are very low, and other at glucose values lower than 2 mM, where SOCR increases dramatically with the decrease in glucose concentration. It is also clear that glutamine enhances SOCR, as can be observed by the higher SOCR observed under the higher glutamine concentration.

Discussion

Glucose metabolism

Glucose uptake

The results obtained show, for BHK cells, the same type of Michaelis-Menten dependence of glucose uptake rate on the glucose concentration that is shown by hybridoma cells (Gaertner and Dhurjati 1993). The values obtained for q_{Glc} range from 1.7 nmol $(10^6 \text{ cells})^{-1}\text{min}^{-1}$ at 0.06 mM glucose to an estimated q_{Glc}^{max} of 32.5 ± 0.8 nmol $(10^6 \text{ cells})^{-1}$ min^{-1}, a somewhat higher value than the reported 13 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} for BHK cells (Neermann and Wagner 1996); these higher values could be due to the fact that they have been obtained under initial-rate kinetics, or to a higher glucose concentration, different media composition or culture systems. The initial-rate kinetics strategy was used to allow comparison of the different glucose uptake rates at an enlarged range of glucose concentrations (0.06–28 mM). The Michaelis-Menten constant K_{Glc}^{Glc} was estimated to be 1.6 ± 0.2 mM, similar to the reported 2 mM for chick embryo fibroblasts (Fagon and Racker 1978), the only K_{Glc}^{Glc} constant available in the literature, since no data have been published for BHK cells.

Lactate production

The estimated q_{Lac}^{max} of 51.6 ± 1.1 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} is larger than the reported lactate production rate of 19 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} for BHK cells (Neermann and Wagner 1996), but is consistent with that obtained in the glucose uptake assay. These results confirm the high dependence of lactate production on the glucose levels in the culture medium.

Glucose to lactate yield

The glucose to lactate yield is essentially constant (around 1.5) for the high glucose concentrations, which is consistent with the value of 1.6 found in the literature (Linz et al. 1997). However, for glucose concentrations below 0.6 mM, the lactate yield decreases dramatically, strongly suggesting a metabolic shift from a high-lactate- to a low-lactate-producing metabolism, extending the reported studies of metabolic changes in hybridoma cells (Zhou et al. 1995). Since a smaller fraction of glucose is being converted to lactate, more efficient pathways, like the complete oxidation of glucose via the tricarboxylic acid cycle, are involved in glucose metabolism as earlier reported for hybridomas (Häggström et al. 1996).

Glucose uptake as a function of glutamine concentration

Glutamine has almost no effect on glucose consumption, but for a marked reduction on glucose consumption observed at very low glutamine concentrations, as observed earlier in hybridoma cells (Ljunggren and Häggström 1994). This reduction may also be due to cell death occurring at very low glutamine concentrations (0.05 mM), as observed in hybridomas (Doverskog et al. 1997).

Influence of glucose concentration on cell growth

The K_m value of glucose for cell growth is lower than 0.8 mM, in agreement with the reported value of 0.5 mM for a hybridoma cell growth (Miller et al. 1988b). Essentially identical growth rates have been obtained under glucose concentrations of 5.5 mM or 0.025 mM, in human diploid fibroblasts (Zielke et al. 1978). Thus, low glucose concentration is a possible tool for manipulating cellular metabolism without any loss in growth performance, which is particularly important if a high cell growth or cell concentration is to be obtained in bioreactions.

Glutamine metabolism

Glutamine uptake

Values of K_{Gln}^{Gln} decreasing from 0.59 ± 0.17 mM to 0.15 ± 0.04 mM glutamine, at glucose concentrations increasing from 0.28 mM to 5.6 mM respectively, were obtained, lower than those often found in the literature: 2 mM in normal rat kidney cells to 4.5 mM in extracts of Ehrlich ascites cells (Xie and Wang 1994), or 4 mM for Madin-Darby canine kidney (MDCK) cells (Glacken et al. 1986). The values obtained for q_{Gln}^{max} of 6.5 ± 0.6 and 2.00 ± 0.11 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} at glucose concentrations of 0.28 mM or 5.6 mM respectively are comparable to those found in the literature: 3.0–4.9 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} (Neermann and Wagner 1996) and 2.4 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} (Fitzpatrick et al. 1993) for BHK cells and 5.7 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} for MDCK cells in medium with fructose instead of glucose (Glacken et al. 1986).

The significantly higher glutamine consumption rate obtained at the lower glucose concentration clearly shows the interactive and complementary nature of both

these main nutrients for animal cells. This glucose/glutamine interaction has been reported for human diploid fibroblasts (Zielke et al. 1978) and also for hybridomas (Häggström et al. 1996; Ljunggren and Häggström 1994; Hu et al. 1987), but was considered to be absent in BHK cells (Linz et al. 1997); this present work confirms that this reciprocal regulation of glycolysis and glutaminolysis is also present in BHK cells. Except at very low glutamine concentrations, the glucose consumption does not change, but the glutamine consumption is significantly increased when the glucose concentration is reduced. This is explained by the fact that, as glutamine is a highly important metabolic building block, its decrease is not compensated by glucose, but the role of glucose as energy source can be compensated by glutamine.

Ammonia production

Previously reported results showed that ammonia production could be reduced (by around 40%) in MDCK cells by maintaining the glutamine concentration at less than 1.0 mM (Glacken et al. 1986). More recently, glutamine concentrations of less than 1.0 mM led to reduced formation rates of ammonium (Linz et al. 1997). In the present work, the values for K_{Amm}^{Gln} of 0.25 ± 0.11 and 0.10 ± 0.03 mM of glutamine, at glucose concentrations of 0.28 mM and 5.6 mM respectively, are lower than those referred to above. However, no real model for ammonia production was proposed in those reports and thus no real comparison can be made. The ammonia production rate was highly dependent not only on the glutamine concentration, but also on the glucose concentration, as already discussed above. The observed increase in the ammonia production with the decrease in glucose concentration was also observed in hybridoma cells (Hu et al. 1987).

Glutamine to ammonia yield

The glutamine to ammonia yield is almost constant for glutamine concentrations above 1 mM. The values obtained (0.7–0.9) are in agreement with those found in the literature for BHK cells (Linz et al. 1997) and also for hybridoma cells (Ozturk and Palsson 1991). However, at low glutamine concentrations (below 1 mM) this yield increased significantly, as a result of the slight increase in the consumption of other amino acids (results not shown) and to a more efficient glutamine metabolism. There are two alternative pathways for the conversion of glutamine to 2-oxoglutarate. One pathway involves glutamate dehydrogenase and releases 2 ammonium and 27 ATP molecules/glutamine molecule; the other pathway involves glutamate:pyruvate transaminase and releases 1 ammonium, 1 alanine and 9 ATP molecules/glutamine molecule (Häggström 1991). At the lower glucose concentration the cell needs to use the glutamine in a more efficient way to compensate for the small amount of glucose available, resulting in an increase in the glutamine to ammonia yield: using glutamine in a more efficient way produces more ATP molecules but, consequently, produces more ammonia per glutamine molecule.

Oxygen consumption

At glucose concentrations lower than 2 mM, there is a dramatic increase in the SOCR with the decrease in glucose concentration, consistent with the metabolic shift at low glucose concentrations already described above: glucose is metabolized through more efficient pathways, with less lactate being produced from glucose, as observed in the glucose to lactate yield presented in Fig. 2.

An additional contribution to the increase in the SOCR with the decrease in glucose concentration is the increase in the glutamine consumption. As glutamine is primarily metabolized through the tricarboxylic acid cycle, it is considered the most important metabolite involved in oxygen consumption (Wohlpart et al. 1990; Zielke et al. 1984). Thus, a decrease in glucose concentration also leads to an increase in glutamine consumption and, consequently, an additional increase in the SOCR. This is also in agreement with the observed increase in the SOCR with the increase in glutamine concentration, for a given constant glucose concentration, as presented in Fig. 6. The values for the SOCR presented in the literature compare well with those obtained in the present study under high glucose concentrations: 0.2 mmol $(10^9 \text{ cells})^{-1}\ h^{-1}$ for BHK cells (Radlett et al. 1972) and 0.1–0.5 mmol $(10^9 \text{ cells})^{-1}\ h^{-1}$ for hybridoma cells (Wohlpart et al. 1990), whereas the present study shows values ranging from 0.05 mmol $(10^9 \text{ cells})^{-1}\ h^{-1}$ at a glucose concentration of 28 mM to 0.7 mmol $(10^9 \text{ cells})^{-1}\ h^{-1}$ at a glucose concentration of 5.5 mM.

The present work quantitatively characterizes, in an integrated way, the metabolism of glucose and glutamine in BHK cells at different nutrient levels, and describes the regulation and interaction of the glucose and glutamine metabolism, proposing a simple model to explain the experimental data. As a conclusion, it can be stated that the glucose and glutamine consumptions and the lactate and ammonia productions show Michaelis-Menten behaviour and that the metabolism at low concentrations of nutrients significantly changes in comparison to that observed at the high levels normally present in the mammalian cell culture media; at low nutrient concentrations, the metabolism is shifted towards more efficient states, where glucose and glutamine are used in a much more effective way and, therefore, the production of lactate and ammonia is markedly reduced.

The experimental approach used in the present work allows the quantitative estimation of the metabolic parameters that will be used to set-up the culture system. This is especially valuable in systems operated in fed-

batch mode, in which cell metabolism is to be confined to a certain state. Similar experimental approaches can be used to study the metabolism of other mammalian cells, in order to establish the strategy for the metabolic optimisation of industrially relevant culture processes.

Acknowledgements The authors acknowledge the financial support received from the European Commission (Biotech BIO2-CT943069 and Biotech ERBIO4-CT960721) and Fundação para a Ciência e a Tecnologia (PBICT/BIO/20333/95 and PRAXIS XXI/BD/2764/94). The technical assistance of Mrs. C.M. Peixoto is also gratefully acknowledged. Fruitful discussion on generic BHK cell physiology with Dr. Sadettin Ozturk from Bayer Corporation, Berkeley, USA, is very gratefully acknowledged.

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