Characterisation of G418-induced metabolic load in recombinant CHO and BHK cells: effect on the activity and expression of central metabolic enzymes

C.A. Yallop^{1,3,*}, P.L. Nørby², R. Jensen¹, H. Reinbach¹ and I. Svendsen¹

¹Department of Cell and Assay Technology, Novo Nordisk A/S, Novo Alle, 2880 Bagsvaerd, Denmark;

²Department of Cloning Technology, Novo Nordisk A/S, Novo Alle, 2880 Bagsvaerd, Denmark; ³Present address: Department of Cell Technology, Crucell Holland BV, Archimedesweg 4, 2333 CN Leiden, The Netherlands; *Author for correspondence (e-mail: C.Yallop@Crucell.com; phone: +31 71 524 8959)

Received 5 December 2002; accepted in revised form 24 April 2003

Keywords: BHK, CHO, Enzymes, G418, Metabolic load, Metabolism, RT-PCR

Abstract

In a previous article (Yallop and Svendsen 2001), recombinant CHO and BHK cell lines, expressing the human glucagon receptor and the gastric inhibitory peptide receptor, respectively, showed reduced growth rates and altered nutrient utilisation when grown with increasing concentrations of G418. This response was associated with an increased expression of the *neo^r* protein, while expression of the recombinant membrane receptors remained unaltered. The metabolic response was characterised in both cell lines by an increase in the specific rate of glutamine utilisation and in CHO cells by a decrease in the yield of lactate from glucose, suggesting a change in the flux of glucose through central metabolism. The aim of this study was to further elucidate these metabolic changes by determining the activity and relative expression of key enzymes involved in glucose and glutamine metabolism. For both CHO and BHK cells, there was an increase in the activity of glutaminase, glutamate dehydrogenase and glutamine synthetase, suggesting an increased flux through the glutaminolysis pathway. The activity of glucose-6-phosphate dehydrogenase and pyruvate carboxylase in CHO cells was also increased whilst lactate dehydrogenase activity remained unaltered, suggesting an increased flux to the pentose phosphate pathway and TCA cycle, respectively. The activity of these enzymes in BHK cells was unchanged. Quantitative RT-PCR showed that expression levels of glutaminase and pyruvate carboxylase were the same with and without G418, indicating that the differences in activities were likely due to post-translational modifications.

Introduction

Recombinant bacteria often show reduced growth rates compared to wild type strains. This is due to an increased metabolic load, caused by the maintenance of high copy number plasmid DNA and high levels of recombinant protein expression (Seo and Bailey 1985; Betenbaugh et al. 1989; Bentley et al. 1990). Recombinant mammalian cells also show signs of an increased metabolic load, typically characterised by a decreased growth rate and an increased rate of nutrient utilisation (Pendse et al. 1992; Gu et al. 1992

1996; Kidane et al. 1997; Schroder and Friedl 1997; Kim et al. 1998). We recently reported that a CHO and BHK cell line containing the neomycin resistance gene (*neo^r*) and expressing the human glucagon receptor (CHO 679) and the gastric inhibitory peptide (BHK 570), showed signs of a metabolic load when G418 was present in the culture medium (Yallop and Svendsen 2001). This was characterised by a reduction in the growth rate and an apparent alteration in central metabolism, indicated by an increase in the cell specific rate of glutamine utilisation and a decrease in the yield of lactate produced from glucose.

In order to further elucidate these changes, a more detailed analysis was initiated. Quantification of central metabolic enzyme activities, using cells grown under different culture conditions, has been used to indicate changes in the flux through metabolic pathways. It has been used to compare glucose and glutamine metabolism in a variety of transformed industrial cell lines (Neerman and Wagner 1996) and to determine the changes in central metabolism as a result of nutrient limitation in hybridoma cells (Vriezen and van Dijken 1998). A similar approach was adopted for this study. Enzymes at key branch points in the major energy-generating central metabolic pathways involving glutamine and glucose were selected and activity and expression levels determined in the presence and absence of G418. By concentrating on these two major pathways, changes in enzyme activity and/or expression levels could also be related to the cell specific rate of nutrient utilisation and production described in the previous article.

Glutamine is a major source of cellular energy, particularly in transformed, immortalised and tumour cells, where it has been implicated in the control of growth, proliferation and apoptosis (Brand 1985; Newsholme et al. 1985; Aledo et al. 1994; Collins et al. 1998; Ko et al. 2001). It is also important in the biosynthesis of amino acids, purine and pyrimidine nucleotides and glucosamine and nicotinamide adenine dinucleotide (Tate and Meister 1973; Stryer 1981). Glutaminolysis, the conversion of glutamine via glutamate to α -ketoglutarate, is a major pathway of glutamine metabolism and a more detailed analysis of this pathway may provide insights into the response of the cells to the G418-induced metabolic stress. As an example, glucose limitation in hybridoma cells has been shown to illicit an increased flux of glutamate through the GDH pathway (Martinelle et al. 1998). In glucose metabolism, changes in the yield coefficient of lactate produced per glucose ($Y_{lac / glc}$) may indicate changes in the flux of glucose through the glycolytic, tricarboxylic acid (TCA) and pentose phosphate (PP) pathways. Details of these changes can give some information on the response of the cells. For example, an increased flux into the TCA cycle via acetyl coA may indicate an increased level of oxidative phosphorylation, while an increased flux via oxaloacetate may indicate an increase in the anapleurotic reactions of the cell, and an increase in the flux to the PP pathway may indicate a demand for reducing equivalents in the form of NADPH. In order to elucidate

Figure 1. A summary of some of the reactions involved in the catabolism of glucose and glutamine. The numbers in the pathway represent some of the important reactions involved in flux control: (1) hexokinase, (2) glucose-6-phosphate dehydrogenase, (3) phosphoenolpyruvate carboxykinase, (4) lactate dehydrogenase, (5) pyruvate carboxylase, (6) pyruvate dehydrogenase complex, (7) citrate synthetase, (8) glutamate dehydrogenase, (9) alanine aminotransferase, (10) aspartate aminotransferase and (11) glutaminase.

some of the changes taking place in the glucose metabolism of these cells, the activity of a number of key glycolytic enzymes were assayed. The enzymes

selected for this study are amongst those highlighted in Figure 1 and were chosen because they represent key regulators or branch points in the pathways of interest. Alterations in their activity may therefore be indicative of alterations in flux through these pathways. The expression level of those enzymes that showed altered activity in the presence of G418 was then measured using real time RT-PCR in order to determine if the changes in enzyme activity were due to an increase in expression or if post-transcriptional mechanisms were important.

Materials and methods

Cell lines and culture media

The stable recombinant cell lines CHO 679, expressing the human glucagon receptor and BHK 570, expressing the human gastric inhibitory peptide (h-GIP) receptor were cloned after transfection of the wild type CHO-K1 and BHK-570 cells as described in Yallop and Svendsen (2001). Cells were grown in a proprietary serum-free medium, SF-1, consisting of a 1:1 mix of DMEM with 4.5 g l⁻¹ glucose and RPMI 1640 (Biowhittaker) plus additional amino acids, fatty acids, vitamins and trace elements. Recombinant human insulin (10 mg l⁻¹, Novo Nordisk), pluronic F-68 (0.3% w/v, Sigma) and penicillin/streptomycin (100 U ml⁻¹/100 mg ml⁻¹, Biowhittaker) were also added. Cells were maintained in 125 ml spinner flasks containing 50 ml media. G418 (Invitrogen) was added to a final concentration of 1 mg ml⁻¹ where appropriate and all cells were kept in logarithmic growth by passage into fresh medium every 1–2 days. Cells were incubated in a humidified incubator at 37 °C and 5% CO₂. Cell numbers were counted using a CASY automatic cell counter (Schärfe Systems). To ensure a single cell suspension prior to counting, 1 ml of cell suspension was incubated with 1 ml of Accumax (Innovative Cell Technologies) at 37 °C for 20 min. Viability was measured using Trypan blue exclusion.

Analytical procedures

Glucose, lactate and ammonia were measured using an Ektachem II analyser (Kodak). Glutamine was measured using a modification of a two-step assay described by Lund (1974). Briefly, the ammonia concentration of the culture supernatant (A_1) was

measured using the Kodak Ektachem II prior to making up the reaction mix. The assay mixture consisted of 0.7 ml of 0.25 M sodium acetate (pH 5.0) containing 0.4 U glutaminase (Sigma) and 0.3 ml of culture supernatant and was incubated for 20 min at 37 °C to ensure complete conversion of glutamine to glutamate. The glutaminase treated solution was then analysed again for ammonia in the Kodak Ektachem II and the result multiplied by 3.33 to take account of the dilution (A_2). The glutamine concentration was calculated by the equation $A_2 - A_1$. A glutamine standard (4 mM) and water blank were always included as controls.

Metabolic parameter calculations

Cell specific rates of substrate utilisation and metabolite production were calculated using the following equations:

$$q_{glc} = [(C_{glc(t1)} - C_{glc(t0)}) \times (1 / N_v)] \times (t1 / t0)$$

$$q_{Lac} = [(C_{lac(t1)} - C_{lac(t0)}) \times (1 / N_v)] \times (t1 / t0)$$

$$q_{Gln} = [(C_{gln(t1)} - C_{gln(t0)}) \times (1 / N_v)] \times (t1 / t0)$$

$$q_{NH4} = [(C_{amm(t1)} - C_{amm(t0)}) \times (1 / N_v)] \times (t1 / t0)$$

where C is the metabolite concentration (μ moles ml⁻¹), $t0$ and $t1$ are the seeding and harvest times, respectively (h) and N_v is the viable cell concentration at $t1$. A rate constant accounting for the spontaneous decomposition of glutamine, as described by Linz et al. (1997) was not included as decomposition was not significant at the time points at which the rates were calculated (data not shown). The molar ratio of the yield of lactate produced per glucose metabolised and ammonia produced per glutamine metabolised were calculated from the equations below:

$$Y_{lac / glc} = q_{lac} / q_{glc}$$

$$Y_{NH4 / gln} = q_{amm} / q_{gln}$$

Cell extracts for enzyme assays

A total of $1-1.5 \times 10^7$ cells were harvested and pelleted by centrifugation at 1000 rpm for 5 min. The pellet was washed twice in cold phosphate-buffered saline and resuspended in an appropriate volume of

cold extraction buffer to a final cell concentration of 1×10^7 cells ml^{-1} . The extraction buffer used for all enzyme assays, except glutamine synthetase, was as described by Irani et al. (1999). It contained 300 mmol l^{-1} sucrose, 100 mmol l^{-1} KCl, 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 10 mmol l^{-1} dithiothreitol (DTT), 0.1% (v/v) triton X-100, 200 $\mu\text{mol l}^{-1}$ leupeptin, 10 $\mu\text{g ml}^{-1}$ aprotinin, 10 $\mu\text{g ml}^{-1}$ phenylmethanesulfonyl fluoride (PMSF) and 50 mmol l^{-1} Tris buffer, pH 7.4. The extraction buffer used for glutamine synthetase was as described by Sanfeliu and Stephanopoulos (1999) and contained 20 mmol l^{-1} imidazole-HCl, pH 7.0, 150 mmol l^{-1} NaCl, 0.02% (v/v) sodium azide, 1.0% (w/v) triton X-100, 0.2 mmol l^{-1} EDTA, 100 $\mu\text{g ml}^{-1}$ PMSF and 1 $\mu\text{g ml}^{-1}$ aprotinin. The cells were then lysed by 45 strokes in a dounce homogenizer and the debris removed by centrifugation at 6000 rpm for 2–3 min. The resulting cell-free extract was either assayed immediately for enzyme activity or frozen at -80°C until required.

Enzyme assays

All enzyme assays were performed in a Hitachi U-2001 spectrophotometer at 25°C . The volume of the reaction mix was 1 ml in all cases and all metabolites were kept on ice during experiments. Endogenous activity was recorded for a minimum of 2 min before starting the reaction. Once started, activity was measured over at least 3 min and the total enzyme activity calculated by subtracting the endogenous activity from the total activity. Enzyme activities were determined in triplicate at three different concentrations of cell-free extract. All enzyme activities were shown to be linearly proportional to the amount of cell-free extract added. Results are presented as nmoles per min per milligram protein, averaged over at least eight determinations. In assays involving NAD/NADH, measurements were taken at 340 nm and the extinction coefficient $E_{340\text{ nm}}$ of $6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used. In assays involving 5,5-dithiobis-2-nitrobenzoic acid (DTNB), measurements were taken at 412 nm and the extinction coefficient $E_{412\text{ nm}}$ of $13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

Hexokinase (E.C. 2.7.1.1) was assayed using the method of Postma et al. (1989a). The assay mixture contained 50 mmol l^{-1} Tris-HCl (pH 8.0), 1 mmol l^{-1} MgCl_2 , 1 mmol l^{-1} NADP, 10 mmol l^{-1} glucose, 0.35 U of glucose-6-phosphate dehydrogenase (Boeh-

ringer Mannheim 127655) and 6 mmol l^{-1} ATP. The reaction was started by the addition of ATP. Pyruvate carboxylase (E.C. 6.4.1.1) was assayed using a malate dehydrogenase coupled reaction according to the method of Payne and Morris (1969). The assay mixture contained 50 mmol l^{-1} Tris-HCl, pH 7.8, 5 mmol l^{-1} ATP, 10 mmol l^{-1} MgCl_2 , 10 mmol l^{-1} KHCO_3 , 2 U malate dehydrogenase (Boehringer Mannheim 127248), 0.1 mmol l^{-1} NADH, 2 mmol l^{-1} sodium pyruvate and 0.5 mmol l^{-1} acetyl CoA. The reaction was started by the addition of pyruvate and acetyl CoA. Lactate dehydrogenase (E.C. 1.1.1.27) was assayed according to the method of Vassault (1983). The assay mixture contained 50 mmol l^{-1} potassium phosphate buffer, pH 7.5, 0.15 mmol l^{-1} NADH and 5 mmol l^{-1} sodium pyruvate. The reaction was started with pyruvate. Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) was assayed according to the method of Postma et al. (1989b). The reaction mixture contained 50 mmol l^{-1} Tris-HCl, pH 7.8, 1 mmol l^{-1} MgCl_2 , 0.4 mmol l^{-1} NADP and 5 mmol l^{-1} glucose-6-phosphate. The reaction was started by the addition of glucose-6-phosphate. Citrate synthetase (E.C. 4.1.3.7) was assayed according to the method of Alp et al. (1976). The reaction mixture contained 50 mmol l^{-1} Tris-HCl, pH 7.8, 0.2 mmol l^{-1} DTNB (5,5-dithio-bis(2-nitrobenzoic acid)), 0.1 mmol l^{-1} acetyl Co A and 0.5 mmol l^{-1} oxaloacetate. The reaction was started by the addition of oxaloacetate. Glutaminase (E.C. 3.5.1.2) was assayed according to the method of Kvamme et al. (1985). The assay mixture contained 100 mmol l^{-1} potassium phosphate buffer, pH 7.8, 5 mmol l^{-1} α -ketoglutarate, 0.2 mmol l^{-1} EDTA, 0.15 mmol l^{-1} NADH, 20 U glutamate dehydrogenase (Boehringer Mannheim 197734) and 10 mmol l^{-1} glutamine. The reaction was started by the addition of glutamine. The chemical decomposition of glutamine was determined in a blank assay without cell-free extract and used to correct measured enzyme activities for any spontaneous decomposition. NAD- and NADP-dependent glutamate dehydrogenase (E.C. 1.4.1.2 and E.C. 1.4.1.4) was assayed according to the method of Schmidt and Schmidt (1983). The reaction mixture contained 50 mmol l^{-1} Tris-HCl, pH 8.5, 1 mmol l^{-1} EDTA, 250 mmol l^{-1} ammonium acetate, 0.15 mmol l^{-1} NADH or NADPH, 1 mmol l^{-1} ADP and 0.4 mmol l^{-1} α -ketoglutarate. The reaction was started by the addition of α -ketoglutarate. Aspartate aminotransferase (E.C. 2.6.1.1) and alanine aminotransferase (E.C. 2.6.1.2) were assayed according to the method of Sugden and

Table 1 Cell specific substrate utilisation, metabolite formation and the molar quotient of the yield of lactate per glucose ($Y_{lac/glc}$) and NH_3 per glutamine utilised ($Y_{amm/gln}$) for CHO 679 cells grown in 125 ml spinners on SF-1 medium with and without 1 mg ml^{-1} G418. Cells were passaged every 1 or 2 days and the concentrations of glucose, lactate, glutamine and NH_3 were determined as described in the materials and methods. Cell specific rates of utilisation and production were calculated for each passage. Each value represent an average of at least 30 measurements made over 2 months. Standard deviation was under 15%.

CHO 679	q_{glc}	q_{lac}	q_{gln}	q_{amm}	$Y_{lac/glc}$	$Y_{amm/gln}$
SF-1+G418	6.13	9.08	1.91	2.02	1.48	1.10
SF-1-G418	6.07	11.00	1.43	1.54	1.81	1.08
BHK 570						
SF-1+G418	9.31	16.92	1.90	1.96	1.82	1.03
SF-1-G418	9.28	16.87	1.45	1.51	1.82	1.04

Newsholme (1975). The reaction mixture for aspartate aminotransferase contained 50 mmol l^{-1} potassium phosphate, pH 7.6, 0.02 mmol l^{-1} pyridoxal phosphate, 0.2 mmol l^{-1} NADH, 10 mmol l^{-1} α -ketoglutarate, pH 7.5, 12 U malate dehydrogenase (Boehringer Mannheim 127248) and 50 mmol l^{-1} aspartate (prepared in KH_2CO_3 , pH 7.6). The reaction mixture for alanine aminotransferase contained 50 mmol l^{-1} potassium phosphate buffer, pH 7.6, 0.2 mmol l^{-1} NADH, 0.02 mmol l^{-1} pyridoxal phosphate, 10 mmol l^{-1} α -ketoglutarate, pH 7.5, 17 U lactate dehydrogenase (Boehringer Mannheim 127221) and 200 mmol l^{-1} alanine. Both reactions were started by the addition of α -ketoglutarate. Glutamine synthetase (E.C. 6.3.1.2) was assayed according to the method of Sanfeliu and Stephanopoulous (1999), based on the formation of glutamyl-hydroxamate from glutamine and hydroxylamine in the presence of ADP. The reaction mixture contained 100 mmol l^{-1} imidazole-HCl, pH 7.2, 50 mmol l^{-1} L-glutamine, 0.4 mmol l^{-1} ADP, 0.2 mmol l^{-1} $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 62.5 mmol l^{-1} hydroxylamine and 10 mmol l^{-1} sodium arsenate. The reaction was started by the addition of $100 \mu\text{l}$ cell-free extract to $400 \mu\text{l}$ of reaction mixture and terminated by the addition of $500 \mu\text{l}$ of ferric chloride solution (370 mmol l^{-1} FeCl_2 and 200 mmol l^{-1} tricarboxylic acid (TCA). Precipitated protein was removed by centrifugation at 9000 rpm for 12 min and the absorbance read at 535 nm against a reagent blank. Negative controls, lacking arsenate, ADP and glutamine were run to ensure that the reaction was a true indication of glutamine synthetase activity.

Protein determination

Protein concentrations of cell-free extracts were de-

termined by the Lowry method using the Dc protein estimation kit (Bio-Rad).

Reverse transcriptase polymerase chain reaction

Total RNA was isolated using the SV total RNA extraction kit (Promega) using the supplied protocol. RNA concentrations were determined by spectrophotometric analysis at 260 nm . Purity was confirmed by analysis at $A_{260 \text{ nm}}/A_{280 \text{ nm}}$. Using the Superscript™ first strand synthesis system (Invitrogen), $1 \mu\text{g}$ of total RNA was reverse transcribed into first strand DNA using oligo(dT) primers. Gene specific primers for pyruvate carboxylase, glutaminase and lactate dehydrogenase (Table 1) were designed from sequence alignments (Vector NTI software, Informax Inc.) using information from human, mouse and rat DNA sequences from the public databases. Oligo 4.05 primer analysis software (National Biosciences Inc.) was used to test selected primers for dimer and duplex formation and to estimate T_m values.

Fragments of the genes for pyruvate carboxylase, glutaminase and lactate dehydrogenase were PCR-amplified from CHO cDNA by Taq polymerase using gene specific primers and cloned in TOPO-TA vectors. Pyruvate carboxylase 2553 bp ; PC-F1: GTGGCCAACAGAGGTGAGATT, PC-R1: CTTGACCTCCTTGAAGTTGGA, Glutaminase; GA-F1: TGATCCCAGGTTGAAAGAGT, GA-R1 769 bp : CCCCAGCAACTCCAGATTTT, Lactate dehydrogenase 947 bp ; LDH-F1: CGGATGAGCTTGCCCTTGTT, LDH-R1: AGATTCCATT-TTGTCCCAGG. The inserts were completely or partly sequenced by the MegaBACE instrument using vector specific primers. The plasmids were used as template for optimisation of LightCycler running conditions and selection of test primers.

Table 2 Cell specific enzyme activities (in nmoles min⁻¹ mg protein⁻¹) of enzymes involved in glucose and glutamine metabolism from CHO 679 and BHK 570 cells grown in SF-1 medium with and without 1 mg ml⁻¹ G418. Activities were determined spectrophotometrically from crude cell extracts as described in materials and methods. Cell extracts were always prepared from 1-day-old cultures. Each value represents an average of at least eight determinations. Standard deviation was not greater than 12%.

Enzyme activity (nmoles min ⁻¹ mg protein ⁻¹)	Cell lines			
	CHO 679		BHK 570	
	-G418	+G418	-G418	+G418
<i>Glutaminolysis</i>				
Alanine aminotransferase	18.1	18.4	17.1	17.3
Aspartate aminotransferase	170.0	171.7	165.5	167.8
Glutamate dehydrogenase	24.0	29.4	22.3	28.1
Glutaminase	348.2	424.8	302.0	378.7
Glutamine synthetase	2.0	3.3	2.2	3.0
<i>Glucose Metabolism</i>				
Hexokinase	102.1	105.4	95.3	97.1
Glucose-6-P dehydrogenase	95.1	114.2	88.6	91.1
Pyruvate carboxylase	1640	2180	1532	1540
Citrate synthetase	104.1	109.6	98.4	99.3
Lactate dehydrogenase	1180	1220	1290	1270

mRNA quantitation

Quantitation of mRNA was performed using a Light-Cycler instrument (Roche). Pyruvate carboxylase primersets used: GACCTGGTCCATGCTCAGATCC/GGGCTCTGCTCATCTTGGTGGC and CACC-ATGCTGGTCAGCTCCCTC/CCAGACTTCATG-GTAGCCGTGC. Glutaminase primersets: AAGCA-GTCTGGAGGGAAGGTT/GCGATGCACATACT-CAGTTCC and GACGTTGGCTAATGGCGGTTT/TTGCAGGAAGACCGACATGGA. Lactate dehydrogenase primerset: CGGATGAGCTTGCCCTTG-TT/AGATTCCATTTTGTCCCAGG. Cyclophilin primerset: CCCCACCGTGTTCTTCGACATCTC/GGTGATCTTCTTGCTGGTTTTvGCC. Light-Cycler run: P1 (denaturation-step); 95 °C for 30 s. P2 (quantification-steps); 95 °C for 0 s, 60 °C for 10 s and 72 °C for 30 s. P3 (melting curve analysis); 95 °C for 0 s, 65 °C for 0 s, 95 °C for 0 s (transition rate: 0.1 °C/s. P4 (cooling); 40 °C for 30 s. The 'Light-Cycler-DNA Master SYBR Green I' kit (Roche) and 4 mM MgCl₂ were used in all experiments. All Light-Cycler data was normalised to the internal control cyclophilin.

mRNA stability

Logarithmic cells cultured in SF-1 medium with and without 1 mg ml⁻¹ G418, were harvested and treated with 0.65 µM 5-6-dichloro-1-β-ribofuranosylben-

zimidazole (DRB), a specific inhibitor of RNA polymerase II transcription (Dubois et al. 1994). At 0, 3, 6 and 9 h post-DRB treatment, total cellular RNA was isolated, reverse transcribed and analysed on the LightCycler as described above.

Results

Metabolic and growth parameters

In order to ensure that any changes in the enzyme activities or expression levels could be directly correlated to the nutrient uptake or production rates, q_{glc} , q_{lac} , q_{gln} and q_{amm} were calculated each day for the cultures that were assayed for enzyme activity and mRNA levels. Table 1 shows that the cultures demonstrated the same trends as observed in the previous study with an increase in the specific rate of glutamine utilisation for CHO and BHK cells (33.6 and 30.0%, respectively) and a decrease in the $Y_{lac/glc}$ for CHO cells (from 1.81 to 1.48). No decrease in $Y_{lac/glc}$ was observed for BHK cells. Glucose utilisation and $Y_{amm/gln}$ for CHO and BHK cells remained unchanged.

Enzyme activities

Glutamine metabolism

The data in Table 2 shows that there was an increase

Table 3 mRNA levels (normalised to cyclophilin) of glutaminase, pyruvate carboxylase and lactate dehydrogenase in CHO 679 and BHK 570 cells grown in SF-1 medium with and without 1 mg ml⁻¹ G418. mRNA was extracted from 1-day-old cell cultures as described in materials and methods. Each value represents an average of at least four experiments carried out in triplicate. Standard deviation was not greater than 15%.

mRNA levels	Cell lines			
	CHO 679		BHK 570	
	-G418	+G418	-G418	+G418
Glutaminase	0.80	0.73	0.70	0.71
Pyruvate carboxylase	0.027	0.024	0.022	0.024
Lactate dehydrogenase	2.73	2.80	2.54	2.42

in the activity of several enzymes of the glutaminolysis pathway. Glutaminase activity increased for both cell-lines in the presence of G418 (22% for CHO 679 and 25.4% for BHK 570), suggesting an increased flux into the glutaminolysis pathway. After the conversion of glutamine to glutamate by glutaminase, two possible routes exist for further conversion to α -ketoglutarate and entry into the TCA cycle, the glutamate dehydrogenase (GDH) route and the transaminase (TA) route, involving for example aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT). For both CHO 679 and BHK 570, the activity of AspAT and AlaAT remained unchanged in the presence of G418. GDH activity however, increased by 22.5% for CHO and 26% for BHK cells, suggesting an increased flux via this route. It might be expected that an increase in GDH activity with no increase in AspAT or AlaAT would result in an increase in $Y_{amm/gln}$ as this reaction yields ammonia. That this was not observed (values of 1.0 ± 0.1 were calculated for both cell lines grown with and without G418) may be due to the increased activity of glutamine synthetase (GS), which increased in both cell-lines in the presence of G418 (65% for CHO 679 and 68% for BHK 570). This increase may be in response to the increased rates of glutamine utilisation and a reduced medium glutamine concentration (Christie and Butler 1999; Sanfeliu and Stephanopoulos 1999) and it may result in an increased conversion of ammonia to glutamine, maintaining $Y_{amm/gln}$ values close to 1.0.

Glucose metabolism

The data in Table 2 shows that for CHO and BHK cells, hexokinase activity was unaltered in the presence of G418, suggesting that flux into glycolysis is unaltered and supporting the earlier findings that G418 did not effect the rate of glucose utilisation. The activity of glucose-6-phosphate dehydrogenase and

pyruvate carboxylase in CHO cells however increased by 20% and 33%, respectively, in the presence of G418, suggesting an increased flux into the pentose phosphate pathway and TCA cycle, respectively. Finally, lactate dehydrogenase activity remained unchanged, suggesting that the shift away from lactate production was not due to a drop in LDH activity. For BHK 570 cells, all the tested enzyme activities were unaltered in the presence of G418, supporting the initial conclusions from the nutrient utilisation data that there were no major changes in the flux through glucose metabolism in response to G418.

Expression levels and mRNA stability

In order to determine if the increase in pyruvate carboxylase and glutaminase activity was a result of increased expression, mRNA levels were measured using LightCycler real time RT-PCR. The expression of lactate dehydrogenase, whose activity did not change in the presence of G418, was tested as a control. Table 3 shows that there was no significant difference in the expression level of any of the enzymes tested. Figure 2 shows that there was also no significant difference in the half-lives of PC and GA mRNA from cells grown with or without G418.

Discussion

Glutamine metabolism

The first step of the glutaminolysis pathway is the conversion of glutamine to glutamate, catalysed by GA. Glutamate can then serve either as a substrate for anabolic pathways, such as amino acid biosynthesis, or can be further metabolised to α -ketoglutarate for entry into the TCA cycle. The increased glutaminase activity observed in this study, together with the

Figure 2. Stability of (A) pyruvate carboxylase, (B) glutaminase and (C) lactate dehydrogenase mRNA isolated from CHO 679 cells grown in SF-1 medium with (closed squares) and without (open squares) G418. mRNA was isolated from logarithmically growing cells 0, 3, 6 and 9 h after incubation with DRB and levels analysed by RT-PCR as described in materials and methods. The data is from one typical experiment where each value represents an average of three determinations. Each experiment was repeated at least three times. Standard deviation between experiments was not greater than 16%.

increased rate of glutamine utilisation, is a strong indicator of an increased metabolism of glutamine in the presence of G418. The increased activity of GDH suggested that there was an increased flux to α -ketoglutarate and the TCA cycle. This change is signifi-

cant as the GDH pathway yields 27 moles of ATP per mole of glutamine consumed, compared to nine ATP for the transaminase pathway (Glacken 1988; Haggstrom 1991). The increase in GDH activity may be due to a decrease in cellular ATP levels as the enzyme activity is increased allosterically in the presence of ADP and inhibited in the presence of ATP (Stryer 1981). Numerous studies have shown that the TA route appears to be the main pathway in continuous cell lines and rapidly proliferating cells. This has been shown both by flux analysis (For example Ardawi and Newsholme 1983; Street et al. 1993; Bonarius et al. 1998) and by measuring enzyme activities (Jenkins et al. 1992; Neerman and Wagner 1996; Vriezen and van Dijken 1998). Within the TA route, AspAT appears to be the major enzyme involved, with AlaAT at a lower level. A similar pattern was also seen in this study, where AspAT showed approximately eight times higher activity than GDH and ten times higher activity than AlaAT. However, the increase in GDH activity in the presence of G418 suggested that the GDH route may be an important pathway for energy production and can be altered under stress conditions. This is supported by Martinelle et al. (1998), who showed that hybridoma cells cultured in an excess of glucose and glutamine metabolize glutamine mainly via the TA route, while in glucose limited conditions, there was an increase in the flux through GDH (and glutaminase). Glutamine synthetase (GS) catalyses the formation of glutamine from free NH_4^+ and glutamate. Its activity is increased under low glutamine conditions and repressed when glutamine is abundant (Kulka and Cohen 1973; Christie and Butler 1999; Sanfeliu and Stephanopoulos 1999). The increased GS activity in this study may be indicative of glutamine limitation as a result of the increased rates of glutamine utilisation.

In this study we have only looked at the major energy-generating pathways of glutaminolysis and glucose metabolism. It would be of interest to look at other pathways such as amino acid metabolism and to determine how these pathways are affected. Another area for future study would be to carry out metabolic flux analysis using ^{15}N and ^{13}C labelled substrates. Changes in enzyme activity have been used to highlight changes in metabolism. Neerman and Wagner (1996) used it to compare the metabolism of a number of different industrial cell lines and Vriezen and van Dijken (1998) to determine the metabolic response of the hybridoma SP2/0 under different culture con-

ditions including glucose limitation. However, enzyme activity data can only be used as an indicator of flux by looking at the ratios between the various enzyme activities and the relative changes in these activities under specific conditions. However, actual flux through the pathways cannot be quantified and the direction of flux cannot be established. The direction of flux is a particular problem because some enzyme reactions are readily reversible under certain conditions, for example flux through GDH was reversed in ammonia stressed hybridoma cells (Bonarius et al. 1998). However, both these issues can be addressed with metabolic flux analysis, using ^{15}N and ^{13}C labelled substrates. For example, it would be interesting to look at the flux through the glutaminolysis pathway as described by Martinelle et al. (1998). The ratio between the GDH and TA pathways under G418 conditions could then be established and used to determine if a greater proportion of the produced glutamate did indeed pass through the GDH pathway as is suggested by the enzyme activity data.

Glucose metabolism

The unaltered hexokinase activity and rate of glucose utilisation suggested that there was no significant change in the flux of glucose entering glycolysis for cells grown with or without G418. The increased activity of pyruvate carboxylase (PC) and glucose-6-phosphate dehydrogenase (G6PDH) in CHO 679 cells suggested an increased flux into the TCA cycle and pentose phosphate pathway, respectively. Pyruvate carboxylase catalyses the carboxylation of pyruvate to oxaloacetate and is a major anapleurotic enzyme involved in the maintenance of the TCA metabolite pool. It is also involved in gluconeogenesis (although this is unlikely to be an important pathway here as glucose was not limiting) and fatty acid synthesis (Stryer 1981). An increase in the activity of PC in response to G418 may therefore indicate an increased flux of pyruvate into the TCA cycle and away from lactate production, thus offering an explanation for the decrease in $Y_{lac/Glc}$ observed in the previous study. PC is a major anapleurotic enzyme in the TCA cycle. The increased activity may therefore be to counter an imbalance in the TCA metabolite pool, perhaps caused by the increased flow of α -ketoglutarate into the TCA cycle from glutamine metabolism. Kight and Fleming (1995) for example, showed that

in small intestine epithelial cells, the fate of pyruvate (derived from glucose or elsewhere) entering the TCA cycle is altered from that of oxidation to anapleurosis in the presence of glutamine. The increased PC activity is therefore a strong indicator of an increased metabolic load. As mentioned, it may also explain the decreased $Y_{lac/Glc}$ observed in this and the previous study. The PC route is not the only entry point of glycolytic metabolites into the TCA cycle and there may additional flux of pyruvate away from lactate via other pathways. For example, pyruvate can also be converted to acetyl coA via the pyruvate dehydrogenase complex, phosphoenolpyruvate to oxaloacetate by phosphoenolpyruvate carboxykinase and pyruvate to malate via the malic enzyme. Of these, we only measured the entry of acetyl CoA into the TCA by measuring citrate synthetase activity and found that the activity of this enzyme was unaltered in the presence of G418. This may indicate that there was no significant increase in the overall flux through the TCA cycle and that the increase in PC activity was therefore most likely to replenish the oxaloacetate pool. Glucose-6-phosphate dehydrogenase is the first reaction of the pentose phosphate pathway and is an important enzyme regulating the biosynthesis of nucleic acid precursors and reducing equivalents in the form of NADPH. It is through the regulation of NADPH, a critical modulator of intracellular redox potential, that G6PDH plays a crucial role in cell growth. Long-term stimulation of the pentose phosphate pathway in general and G6PDH activity in particular is known to occur in response to growth factor addition and in parallel with proliferation (Stanton and Seifter 1988; Stanton et al. 1991; Tian et al. 1994). A more direct involvement was demonstrated by Tian et al. (1998) who showed that over-expression of G6PDH in 3T3 and COS-7 cells resulted in growth stimulation, while inhibition abrogated growth factor stimulation and increased sensitivity to the inhibitory effects of H_2O_2 . G6PDH is also essential for resistance to oxidative stress (Salvemini et al. 1999). The increase in G6PDH activity observed in this study in response to G418 suggested an increased flux into the pentose phosphate pathway. This may represent an increased demand for reducing equivalents, it may be required as a response to increased oxygen radical production during oxidative metabolism (for example H_2O_2 produced during the GDH reaction) and it may indicate an increased demand for nucleic acid precursors in response to

increased DNA/RNA synthesis. Conversely, the unaltered activity of PC and G6PDH in BHK 570 cells grown with and without G418 supported the nutrient utilisation data and suggested that these cells were not able to alter their metabolism in the same way as the CHO cells. The failure to do this may be a contributing factor to the relatively poor behaviour of these cells in the presence of G418 compared to CHO 679 (Yallop and Svendsen 2001).

Metabolic responses to G418

Taken together, the enzyme activity data suggested that growth in the presence of G418 results in significant alterations in the metabolism of the cells with an increased flux through key central metabolic pathways. It clearly supports the nutrient utilisation data of the earlier study and suggests that growth in the presence of G418 resulted in an increased metabolic load. Moreover, it seems to suggest that the CHO cells were more flexible in their ability to alter their metabolism than the BHK cells and that this greater flexibility may have resulted in the relatively better growth of these cells. However, flux analysis is needed to quantify and verify these changes and additional pathways such as those involved in amino acid metabolism need to be studied.

Enzyme regulation and expression analysis

As with many metabolic enzymes, pyruvate carboxylase is subject to long-term regulation at the level of transcription. For example, glucose starvation and differentiation in rat and human cells resulted in increased mRNA levels (Freytag and Utter 1983; Zhang et al. 1995; Salto et al. 1996 1999). It is also subject to short-term, post-transcriptional regulation, for example activity is increased allosterically by the positive effector acetyl-CoA (Barrit et al. 1966) and by high concentrations of pyruvate (McClure and Lardy 1971) and is inhibited by high levels of ATP. The fact that the levels of CHO PC mRNA remained unaltered in the presence of G418 whilst activity increased, suggests that post-transcriptional regulation may be important and that alteration in mRNA stability is not the mechanism involved.

An additional mechanism may be the formation of multiple transcripts. Multiple PC mRNA transcripts have been identified in rats, mice and humans, that have the same coding region but which diverge at

their 5' untranslated region (Jitrapakdee et al. 1996 2001). This heterogeneity is generated by alternative splicing, with different isoforms expressed in different tissues. In rats for example, two promoters are responsible for the production of two primary transcripts that are then alternately spliced into five mature transcripts with different 5'-UTRs (Jitrapakdee et al. 1997). Transcripts from the proximal promoter were highly transcribed in gluconeogenesis and lipogenesis, whereas transcripts from the distal promoter were necessary for anapleurosis (Jitrapakdee et al. 1998). The different transcripts also showed different translational efficiencies, suggesting an additional regulation mechanism and a possible effect of the 5' UTR heterogeneity (Jitrapakdee et al. 1998). If multiple transcripts are produced in transformed hamster cells, one explanation for the increased activity may be an increase in the levels of one transcript (for example one involved in anapleurosis), combined with a decrease in another transcript(s) such that there was no overall change in PC mRNA levels. To establish this we plan to quantify the levels specific transcripts by designing primers to the 3' and 5' UTR. Another explanation is that there is a change in the translation efficiency of the produced transcript induced by the culture conditions. A final possibility is that allosteric regulation by metabolites such as acetyl CoA leads to a short-term increase in activity. In rats, two types of glutaminase have been identified, hepatic and kidney type. These are the products of separate genes and several isoforms have been identified for each type, formed by tissue-specific alternative splicing. This post-transcriptional processing may regulate the expression of GA activity in certain tissues by the production of transcripts with different activities (Labow et al. 2001). The level of renal GA mRNA is also regulated in response to starvation by a mechanism that does not involve transcriptional or translational regulation (Hwang and Curthoys 1991; Hwang et al. 1991). Two isoforms of renal GA are expressed, which contain a pH responsive element in the 3' untranslated region that regulates GA mRNA levels by increasing mRNA stability in response to acidosis (Hansen et al. 1996). The stability is modified in response to acidosis by increased binding of a pH-response element binding protein (REBP) to the pH-response element in the 3' UTR of the GA mRNA (Laterza et al. 1997; Laterza and Curthoys 2000). As with PC, the fact that the levels of GA mRNA were unaltered in the presence of G418, whilst activity

increased suggested that post-transcriptional regulation was important. Kong et al. (1998) reported a similar situation in enterocytes isolated from the ileum and colonocytes isolated from the colon of rats. Both cell types contained similar levels of GA mRNA, whilst there was a large difference in activity between the two cell types. However, altered mRNA stability appeared not to be the mechanism involved in this case. Obviously, a more detailed analysis of the expression of these two enzymes is required, particularly to establish if multiple transcripts are produced, if there is a change in the ratio of these transcripts and if there is a change in the translation efficiency in the presence of G418. However, from the data we have collected, it appears likely that G418 elicits a short-term response in the cells involving some form of post-transcriptional regulation of key metabolic enzymes. As mentioned, post-transcriptional mechanisms are known to play an important role in the regulation of other central metabolic enzymes and it is likely that similar mechanisms are responsible for the regulation of G6PDH and GDH. For example GDH is strongly regulated by ADP and ATP levels (Stryer 1981), while G6PDH activity is regulated by altered splicing, mRNA stability and release from a structural element within the cell (Stanton et al. 1991; Amir-Ahmady and Salati 2001). It is planned to look at the expression of G6PDH and GDH along with GA and PC in order to gain a more comprehensive insight into how these enzymes are regulated in response to G418-induced metabolic load.

Uncited reference

Lopez-Casillas and Kim 1991

References

- Aledo J.C., Segura J.A., Medina M.A., Alonso F.J., de Castro I.N. and Marquez J. 1994. Phosphate-activated glutaminase expression during tumor development. *FEBS Lett.* 341: 39–42.
- Alp P.R., Newsholme E.A. and Zammit V.A. 1976. Activities of citrate synthase and NAD-linked and NADP-linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates. *Biochem. J.* 154: 689–700.
- Amir-Ahmady B. and Salati L.M. 2001. Regulation of the processing of glucose-6-phosphate dehydrogenase mRNA by nutritional status. *J. Biol. Chem.* 276: 10514–10523.
- Ardawi M.S.M. and Newsholme E.A. 1983. Glutamine metabolism in lymphocytes of the rat. *Biochem. J.* 212: 835–842.
- Barrit G.J., Keech D.B. and Ling A.M. 1966. Apparent cooperative effect of acetyl-CoA on sheep kidney pyruvate carboxylase. *Biochem. Biophys. Res. Commun.* 24: 476–481.
- Bentley W.E., Mirjalili N., Andersen D.C., Davis R.H. and Kompala D.S. 1990. Plasmid-encoded protein: The principal factor in the 'metabolic burden' associated with recombinant bacteria. *Biotechnol. Bioeng.* 35: 668–681.
- Betenbaugh M.J., Beaty C. and Dhurjati P. 1989. Effects of plasmid amplification and recombinant gene expression on the growth kinetics of recombinant *Escherichia coli*. *Biotechnol. Bioeng.* 33: 1425–1436.
- Bonarius H.P.J., Houtman J.H.M., de Gooijer C.D., Tramper J. and Schmid G. 1998. Activity of glutamate dehydrogenase is increased in ammonia-stressed hybridoma cells. *Biotechnol. Bioeng.* 57: 447–453.
- Brand K. 1985. Glutamine and glucose metabolism during thymocyte proliferation: Pathways of glutamine and glutamate metabolism. *Biochem. J.* 228: 353–361.
- Christie A. and Butler M. 1999. The adaptation of BHK cells to a non-ammoniogenic glutamate-based culture medium. *Biotechnol. Bioeng.* 64: 298–309.
- Collins C.L., Wasa M., Souba W.W. and Abcouwer S.F. 1998. Determinants of glutamine dependence and utilisation by normal and tumor-derived breast cell lines. *J. Cell Physiol.* 176: 166–178.
- Dubois M.F., Nguyen V.T., Bellier S. and Bensuade O. 1994. Inhibitors of transcription such as 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole and isoquinoline sulphonamide derivatives (H8 and H7) promote dephosphorylation of the carboxyl terminal domain of RNA polymerase II largest sub-unit. *J. Biol. Chem.* 269: 13331–13336.
- Freytag S.O. and Utter M.F. 1983. Regulation of the synthesis and degradation of pyruvate carboxylase in 3T3-L1 cells. *J. Biol. Chem.* 258: 6307–6312.
- Glacken M.W. 1988. Catabolic control of mammalian cell culture. *Bio/Technology* 6: 1041–1050.
- Gu M.B., Kern J.A., Todd P. and Kompala D.S. 1992. Effect of amplification of *dhfr* and *lacZ* genes on growth and β -galactosidase expression in suspension cultures of recombinant CHO cells. *Cytotechnology* 9: 237–245.
- Gu M.B., Todd P. and Kompala D.S. 1996. Metabolic burden in recombinant CHO cells: effect of *dhfr* gene amplification and *lacZ* expression. *Cytotechnology* 18: 159–166.
- Haggstrom L. 1991. Energetics of glutaminolysis – a theoretical evaluation. In: Spier R.E., Griffiths J.B. and Meignier B. (eds), *Production of Biologicals from Animal Cells in Culture*. Butterworth-Heinemann Ltd., Oxford, pp. 79–81.
- Hansen W.R., Barsic-Tress N., Taylor L. and Curthoys N.P. 1996. The 3'-non-translated region of rat renal glutaminase mRNA contains a pH-responsive stability element. *Am. J. Physiol.* 271: F126–F131.
- Hwang J.-J. and Curthoys N.P. 1991. Effect of acute alterations in acid-base balance on rat renal glutaminase and phosphoenolpyruvate carboxykinase gene expression. *J. Biol. Chem.* 266: 9392–9396.
- Hwang J.-J., Perera S., Shapiro R.A. and Curthoys N.P. 1991. Mechanism of altered renal glutaminase gene expression in response to chronic acidosis. *Biochemistry* 30: 7522–7526.
- Irani N., Wirth M., van den Heuvel J. and Wagner R. 1999. Improvement of the primary metabolism of cell cultures by

- introducing a new cytoplasmic pyruvate carboxylase reaction. *Biotechnol. Bioeng.* 86: 238–246.
- Jenkins H.A., Butler M. and Dickson A.J. 1992. Characterisation of glutamine metabolism in two related murine hybridomas. *J. Biotechnol.* 23: 167–182.
- Jitrapakdee S., Walker M.E. and Wallace J.C. 1996. Identification of novel alternatively spliced pyruvate carboxylase mRNAs with divergent 5'-untranslated regions, which are expressed in a tissue-specific manner. *Biochem. Biophys. Res. Comm.* 223: 695–700.
- Jitrapakdee S., Booker G.W., Cassady A.I. and Wallace J.C. 1997. The rat pyruvate carboxylase gene structure: Alternate promoters generate multiple transcripts with 5'-end heterogeneity. *J. Biol. Chem.* 272: 20522–20530.
- Jitrapakdee S., Gong Q., MacDonald M.J. and Wallace J.C. 1998. Regulation of rat pyruvate carboxylase gene expression by alternate promoters during development in genetically obese rats and in insulin secreting cells. *J. Biol. Chem.* 273: 34422–34428.
- Jitrapakdee S., Petchampai N., Sunyakumthorn P., Wallace J.C. and Boonsaeng V. 2001. Structural and promoter regions of the murine pyruvate carboxylase gene. *Biochem. Biophys. Res. Commun.* 287: 411–417.
- Kidane A.H., Guan Y., Evans P.M., Kaderbhai M.A. and Kemp R.B. 1997. Comparison of heat flux in wild type and genetically engineered chinese hamster ovary cells. *J. Thermal Analysis* 49: 771–783.
- Kight C.E. and Fleming S.E. 1995. Oxidation of glucose carbon entering the TCA cycle is reduced by glutamine in small intestine epithelial cells. *Am. J. Physiol.* 268: G879–G888.
- Kim S.J., Kim N.S., Ryu C.J., Hong H.J. and Lee G.M. 1998. Characterisation of chimeric antibody-producing CHO cells in the course of dihydrofolate reductase-mediated gene amplification and their stability in the absence of selective pressure. *Biotechnol. Bioeng.* 58: 73–84.
- Ko Y.-G., Kim E.-K., Kim T., Park H.-S., Choi E.-J. and Kim S. 2001. Glutamine-dependent antiapoptotic interaction of human glutaminyl-tRNA synthetase with apoptosis signal regulating kinase 1. *J. Biol. Chem.* 276: 6030–6036.
- Kong S.-E., Heel K.A., Hall J.C. and McCauley R.D. 1998. Quantitation of glutaminase mRNA in enterocytes using competitive RT-PCR. *Mol. Cell Probes* 12: 339–341.
- Kulka R.G. and Cohen H. 1973. Regulation of glutamine synthetase activity of hepatoma tissue culture cells by glutamine and dexamethasone. *J. Biol. Chem.* 248: 6738–6743.
- Kvamme E., Torgner I.A. and Svenneby G. 1985. Glutaminase from mammalian tissues. *Methods Enzymol.* 113: 241–256.
- Labow B.I., Souba W.W. and Abcouwer S.F. 2001. Mechanisms governing the expression of the enzymes of glutamine metabolism—glutaminase and glutamine synthetase.
- Laterza O.F., Hansen W.R., Taylor L. and Curthoys N.P. 1997. Identification of an mRNA-binding protein and the specific elements that may mediate the pH-responsive induction of renal glutaminase mRNA. *J. Biol. Chem.* 272: 22481–22488.
- Laterza O.F. and Curthoys N.P. 2000. Effect of acidosis on the properties of the glutaminase mRNA pH-response element binding protein. *J. Am. Soc. Nephrol.* 11: 1583–1588.
- Linz M., Zeng A.-P., Wagner R. and Deckwer W.-D. 1997. Stoichiometry, kinetics and regulation of glucose and amino acid metabolism of a recombinant BHK cell line in batch and continuous culture. *Biotechnol. Prog.* 13: 453–463.
- Lopez-Casillas F. and Kim K.-H. 1991. The 5' untranslated regions of acetyl coenzyme A carboxylase mRNA provide specific translational control *in vivo*. *Eur. J. Biochem.* 201: 119–127.
- Lund P. 1974. Glutamine: Determination with glutaminase and glutamate dehydrogenase. In: Bergmeyer H.U. (ed.), *Methods of Enzymatic Analysis*. Vol. 4. Academic Press, London, pp. 1719–1722.
- Martinelle K., Doverskog M., Jacobsen U., Chapman B.E., Kuchel P.W. and Haggstrom L. 1998. Elevated glutamate dehydrogenase flux in glucose-deprived hybridoma and myeloma cells: evidence from $^1\text{H}/^{15}\text{N}$ NMR. *Biotechnol. Bioeng.* 60: 508–517.
- McClure W.R. and Lardy H.A. 1971. Rat liver pyruvate carboxylase IV: factors that affect the regulation *in vivo*. *J. Biol. Chem.* 246: 3591–3596.
- Neerman J. and Wagner R. 1996. Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell lines, insect and primary liver cells. *J. Cell Physiol.* 166: 152–169.
- Newsholme E.A., Crabtree B. and Ardawi M.S.M. 1985. The role of high rates of glycolysis and glutamine utilisation in rapidly dividing cells. *Biosci. Rep.* 5: 393–400.
- Payne J. and Morris J.G. 1969. Pyruvate Carboxylase in *Rhodospseudomonas spheroides*. *J. Gen. Microbiol.* 59: 97–101.
- Pendse G.J., Karkare S. and Bailey J.E. 1992. Effect of cloned gene dosage on cell growth and hepatitis B surface antigen synthesis and secretion in recombinant CHO cells. *Biotechnol. Bioeng.* 40: 119–129.
- Postma E., Scheffers W.A. and van Dijken J.P. 1989. Kinetics of growth and glucose transport in glucose limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066. *Yeast* 5: 159–165.
- Postma E., Verduyn C., Scheffers W.A. and van Dijken J.P. 1989. Enzymic analysis of the Crabtree effect in glucose-limiting chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 55: 468–477.
- Salto R., Sola M., Oliver F.J. and Vargas A.M. 1996. Effects of starvation, diabetes and carbon tetrachloride intoxication on rat kidney cortex and liver pyruvate carboxylase levels. *Arch. Physiol. Biochem.* 104: 845–850.
- Salto R., Giron M.D., Sola M. and Vargas M. 1999. Evolution of pyruvate carboxylase and other biotin containing enzymes in developing rat liver and kidney. *Mol. Cell Biochem.* 200: 111–117.
- Salvemini F., Franze A., Iervolino A., Filosa S., Salzano S. and Ursini M.V. 1999. Enhanced glutathione levels and oxidoresistance mediated by increased glucose-6-phosphate dehydrogenase expression. *J. Biol. Chem.* 274: 2750–2757.
- Sanfeliu A. and Stephanopoulos G. 1999. Effect of glutamine limitation on the death of attached Chinese Hamster Ovary cells. *Biotechnol. Bioeng.* 64: 46–53.
- Schmidt E. and Schmidt F.W. 1983. Glutamate dehydrogenase. In: Bergmeyer H.U. (ed.), *Methods of Enzymatic Analysis*. Vol. 3. Academic Press, New York, pp. 216–227.
- Schroder M. and Friedl P. 1997. Overexpression of recombinant human antithrombin III in Chinese hamster ovary cells results in malformation and decreased secretion of recombinant protein. *Biotechnol. Bioeng.* 53: 547–559.
- Seo J.-H. and Bailey J.E. 1985. Effects of recombinant plasmid content on growth properties and cloned gene product formation in *Escherichia coli*. *Biotechnol. Bioeng.* 27: 1668–1674.
- Stanton R.C. and Seifter J.L. 1988. Epidermal growth factor rapidly

- activates the hexose monophosphate shunt in kidney cells. *Am. J. Physiol.* 254: C267–C271.
- Stanton R.C., Seifter J.L., Boxer D.C., Zimmerman E. and Cantley L.C. 1991. Rapid release of bound glucose-6-phosphate dehydrogenase by growth factors. *J. Biol. Chem.* 266: 12442–12448.
- Street J.C., Delort A.-M., Braddock P.S.H. and Brindle K.M. 1993. A $^1\text{H}/^{15}\text{N}$ NMR study of nitrogen metabolism in cultured mammalian cells. *Biochem. J.* 291: 485–492.
- Stryer L. 1981. *Biochemistry*. 2nd edn. WH Freeman and Company, New York.
- Sugden P.M. and Newsholme E.A. 1975. Activities of citrate synthase, NAD-linked and NADP-linked isocitrate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase in nervous tissue from vertebrates and invertebrates. *Biochem. J.* 150: 105–111.
- Tate S.S. and Meister A. 1973. In: Prusiner S. and Stadtman E.R. (eds), *The Enzymes of Glutamine Metabolism*. Academic Press, New York, pp. 77–125.
- Tian W.-N., Pignatere J.N. and Stanton R.C. 1994. Signal transduction proteins that associate with the platelet-derived growth factor (PDGF) receptor mediate the PDGF-induced release of glucose-6-phosphate dehydrogenase from permeabilised cells. *J. Biol. Chem.* 269: 14798–14805.
- Tian W.-N., Braunstein L.D., Pang J.-D., Stuhlmeier K.M., Xi Q.-X., Tian X. and Stanton R.C. 1998. Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *J. Biol. Chem.* 273: 10609–10617.
- Vassault A. 1983. Lactate dehydrogenase, UV method with pyruvate and NADH. In: Bergmeyer H.U. (ed.), *Methods of Enzymatic Analysis*. Vol. 3. Academic Press, New York, pp. 118–126.
- Vriezen N. and van Dijken J.P. 1998. Fluxes and enzyme activities in central metabolism of myeloma cells grown in chemostat culture. *Biotechnol. Bioeng.* 59: 28–39.
- Yallop C.A. and Svendsen I. 2001. The effects of G418 on the growth and metabolism of recombinant mammalian cell lines. *Cytotechnology* 35: 101–114.
- Zhang J., Xia W.-L. and Ahmad F. 1995. Regulation of pyruvate carboxylase in 3T3-L1 cells. *Biochem. J.* 306: 205–210.