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The effect of glucose and glutamine on the intracellular nucleotide pool and oxygen uptake rate of a murine hybridoma

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Abstract

The effects of media concentrations of glucose and glutamine on the intracellular nucleotide pools and oxygen uptake rates of a murine antibody-secreting hybridoma cell line were investigated. Cells taken from mid-exponential phase of growth were incubated in medium containing varying concentrations of glucose (0–25 mM) and glutamine (0–9 mM). The intracellular concentrations of ATP, GTP, UTP and CTP, and the adenylate energy charge increased concomitantly with the medium glucose concentration. The total adenylate nucleotide concentration did not change over a glucose concentration range of 1–25 mM but the relative levels of AMP, ADP and ATP changed as the energy charge increased from 0.36 to 0.96. The maximum oxygen uptake rate (OUR) was obtained in the presence of 0.1–1 mM glucose. However at glucose concentrations >1 mM the OUR decreased suggesting a lower level of aerobic metabolism as a result of the Crabtree effect. A low concentration of glutamine (0.5 mM) caused a significant increase (45–128%) in the ATP, GTP, CTP, UTP, UDP-GNac, and NAD pools and a doubling of the OUR compared to glutamine-free cultures. The minimal concentration of glutamine also caused an increase in the total adenylate pool indicating that the amino acid may stimulate the *de novo* synthesis of nucleotides. However, all nucleotide pools and the OUR remained unchanged within the range of 0.5–9 mM glutamine. Glucose was shown to be the major substrate for energy metabolism. It was estimated that in the presence of high concentrations of glucose (10–25 mM), glutamine provided the energy for the maintenance of up to 28% of the intracellular ATP pool, whereas the remainder was provided by glucose metabolism.

Introduction

The maintenance of intracellular nucleotide pools is essential for the viability of mammalian cells in culture. Some evidence suggests that specific nucleotide concentration ratios can be used as coefficients of productivity from protein producer cell lines (Ryll and Wagner, 1992; Barnabé and Butler, 1998). The intracellular nucleotide pools are dependent upon the ability of cells to derive energy from available nutrients.

The two major nutrients in cell culture are glucose and glutamine. Their metabolism has been well-characterized for antibody-secreting hybridomas (Butler and Jenkins, 1989; Miller et al., 1989a, b). Both substrates are fuels for energy metabolism and are precursors for biomass synthesis. Glucose which is normally present in culture medium at a concentration of 5–25 mM provides a majority of its energy through aerobic glycolysis (Fitzpatrick et al., 1993; Petch and Butler, 1994). This energy yielding pathway, a characteristic of transformed cell lines, converts glucose to lactate even though oxygen is present (Medina and Nunez de Castro, 1990). Glucose also supplies energy via the tricarboxylic acid (TCA) cycle and the pentose phosphate pathway, the latter being an important supplier of nucleic acid precursors (Zielke et al., 1976; Reitzer et al., 1980).

Glutamine is normally supplied in culture medium at a concentration of 1–6 mM, which is significantly higher than the other amino acids. Glutamine provides energy via glutaminolysis which involves the

complete or partial oxidation of the substrate via the TCA cycle. Complete oxidation leads to CO_2 whereas partial oxidation leads to 3 or 4 carbon metabolites which include aspartate, lactate or alanine. The partial breakdown of glutamine has been reported in many transformed cell lines (McKeehan, 1982; Lanks and Li, 1988) and hybridomas (Fitzpatrick et al., 1993; Petch and Butler, 1994). Glutamine is also required as a precursor for nucleic acid synthesis (Engstrom and Zetterberg, 1984).

Estimates of energy production from glucose and glutamine have been based previously on end product analysis of substrates and oxygen consumption rates using assumed stoichiometric ratios to determine ATP yields (Fitzpatrick et al., 1993; Petch and Butler, 1994; Meijer and Van Dijken, 1995). These studies however do not take into account actual intracellular levels of ATP or other nucleotides in the cell which are subject to a balance between energy requiring processes, energy providing processes and *de novo* nucleotide synthesis. The approach adopted in the experiments reported here was to determine the contribution of the carbon substrates to energy metabolism from direct measurements of nucleotide concentrations in the cell. Understanding energy production and nucleotide pool maintenance in hybridomas can form the basis for the development of control strategies such as nutrient feeding which would ensure sufficient energy is available for maintaining culture viability and high antibody production.

The hybridoma used in this work was the well-characterised murine B-lymphocyte CC9C10 line which secretes an antibody (IgG) against bovine insulin. The experiments were designed to determine the intracellular nucleotide profiles of the hybridoma during exposure to varying extracellular concentrations of glucose and glutamine. The analysis provided information regarding the relative contribution of the two main substrates to the cellular energy metabolism.

Materials and methods

Cell line and media

The murine B-lymphocyte hybridoma (CC9C10) which secretes a monoclonal antibody against bovine insulin was obtained from the American Type Culture Collection (ATCC No. HB123). The cells were grown in a defined serum-free medium (NB-SFM) at 37 °C under a 10% CO_2 humidified atmosphere (Barnabé and Butler, 1994). The media used in stock cultures contained 25 mM glucose and 6 mM glutamine. The concentrations of these substrates were varied during experimental procedures. All chemicals were purchased from the Sigma Chemical Co. unless otherwise noted.

Culture conditions

Preliminary experiments indicated that the intracellular nucleotide concentrations of the cells varied significantly and was dependent upon the protocol of sub-culture. To minimize variability in the state of the cell inoculum, stock cultures were treated in a strict regimen prior to use in experiments. Cells were inoculated at 1.0×10^5 cells/ml in T-flasks (150 cm^2) containing 50 ml growth medium. After 46 h of incubation at 37 °C, 10 ml of the culture was removed and replaced with an equivalent volume of fresh medium. Cultures were then incubated for another 21–25 h until the cell density reached $0.8–1.0 \times 10^6$ cells/ml. The glucose and glutamine concentrations in these cultures were 11.1 ± 0.6 and 3.3 ± 0.4 mM, respectively. The cells which were now in the mid-exponential phase of growth were collected by centrifugation at 190 g for 5 min. The supernatant was decanted and the cells were washed once in the medium to be used in the experiment. The cells were centrifuged and resuspended at 5×10^5 cells/ml in 11 ml experimental medium in a T-flask (25 cm^2) and incubated at 37 °C. The glucose and glutamine concentration in the experimental medium was varied from 0–25 and 0–9 mM, respectively.

Cell counting and metabolite assays

Viable cell concentrations were determined by counting a trypan blue diluted cell suspension on a Neubauer haemocytometer. Glucose concentrations were determined with a glucose analyzer (Model YSI 27, Yellow Springs Instrument Co.). Glutamine was measured using a modified enzymatic assay (Lund, 1985) and a 96 well plate reader (Thermomax by Molecular Devices).

Analysis of nucleotide pools

Nucleotides were extracted from cells after 5 h of incubation in each medium. Hybridoma cells (1.25×10^6 cells) were sedimented at 350 g for 8 min. Cell pellets were resuspended in 150 μl of ice cold 6% trichloroacetic acid, sonicated for 15 s. and kept

on ice for 15 min. The suspension was subsequently sedimented at 4 °C and 14 000g for 10 min. The supernatants were neutralized and stored at –20 °C. Nucleotides were separated by HPLC with a C_{18} column (200 mm × 4.6 mm I.D., 5 μm particle size, Alltech) as described previously (Barnabé and Butler, 1994). Nucleotide peaks were identified and quantified by comparing their retention times and peak areas (254 nm) with those of standard mixtures. Chromatograms were analyzed with EZChrom v.3.2 software (Shimadzu). As the column aged, adenine nucleotide peaks fused with other nucleotide peaks. In this case, a bioluminescent assay was used to determine the concentration of adenine nucleotides (Lundin et al, 1986). Eleven nucleotides were quantified with these two methods: NAD, UDP-Glc, UDP-GalNac, UDP-GlcNac, AMP, GDP, CTP, ADP, UTP, GTP and ATP.

Oxygen uptake rate (OUR)

OUR of cells was measured after 3–5 h of incubation at 37 °C in media containing variable concentrations of glucose and glutamine. The OUR was determined by the rate of decrease of dissolved oxygen concentration using a Biological Oxygen Monitor (YSI Model 5300), oxygen probe (YSI Model 5331) and a chart recorder (ABB Goerz AG). The probe was inserted into a YSI glass chamber containing 10 ml of cell suspension (5×10^5 cells/ml) and a 20 mm Teflon coated magnetic stir bar, making sure all the air was removed from the chamber. The assembly was put in a 37 °C stirred water bath containing a submergible stir plate set at 700 rpm (Wohlpart et al., 1990). Oxygen depletion was monitored for 15 min. The OUR was calculated assuming an oxygen concentration of 0.22 mM at air saturation.

Statistical analysis

Tests of significant difference were computed by analysis of variance (ANOVA) using Sigmaplot software.

Results

Effect of glucose and glutamine on the ATP nucleotide pool

Cells taken from stock cultures at the mid-exponential phase of growth were inoculated into culture medium containing varying concentrations of glucose (0–25 mM) and glutamine (0–9 mM). The concentrations of the intracellular nucleotide pools were determined after a 5 h incubation period.

Table 1 shows the intracellular ATP concentrations of cells as analyzed by HPLC. Cultures without glutamine at low levels of glucose (0–3 mM) were not included in this matrix because the formulated media were not commercially available. The data show a clear trend that an increase in the glucose concentration from 0 to 25 mM resulted in a concomitant increase in the intracellular ATP pool regardless of the glutamine concentration. ($p < 0.005$). The initial ATP concentration of the cells prior to incubation was 3.98 ± 0.59 nmol/10^6 cells . Thus, the data show that the intracellular ATP pool decreased during incubation in media containing glucose below 10 mM and glutamine below 0.5 mM. Not surprisingly these substrate concentrations were lower than those in the culture from which the cell inoculum was taken (11.1 mM glucose, 3.3 mM glutamine).

The presence of 0.5 mM glutamine at the higher glucose concentrations (10–25 mM) caused a significant increase in ATP. The maximum intracellular ATP was determined as 6.25 nmol/10^6 cells in cells incubated in media containing 25 mM glucose and >0.5 mM glutamine. Increasing the media glutamine concentration from 0.5 to 9 mM did not significantly alter the intracellular ATP pool.

Assuming that glucose and glutamine were the major carbon substrates of cellular catabolism leading to ATP formation, the relative contribution of each substrate to the intracellular ATP concentration can be calculated. At 10 and 25 mM glucose, the effect of adding glutamine (>0.5 mM) was to increase the intracellular ATP level by 0.89 and 1.76 nmol/10^6 cells, respectively . There was no significant increase in the ATP level when the glutamine concentration was increased from 0.5 to 9 mM. From this data the effect of glutamine could be calculated as representing 17.3 and 28.1% of the maximum measured increase in ATP at 10 and 25 mM glucose, respectively.

The effect of glucose and glutamine on the adenylate energy charge

The intracellular energy charge is an index of the state of phosphorylation of the adenylate nucleotides in the cell. The index is an indication of the metabolic status of the cell, which tends to a high value (theoretical maximum = 1) following a high level of catabolic activity. The effect of the carbon substrates on the intracellular adenylate energy charge was determined in

Table 1. The effect of glucose and glutamine concentration on the intracellular ATP pool (nmol/10^6 cells). Hybridomas were inoculated into T-flasks (25 cm^2) containing 11 ml NB-SFM at varying glucose and glutamine concentrations. Intracellular nucleotides were extracted after 5 h of incubation at 37 °C. The initial ATP value of stock cultures was 3.98±0.59 nmol/10^6 cells/ml (n = 3)

Glutamine (mM)	Glucose (mM)					
	0	1	3	5	10	25
0	ND	ND	ND	3.20±0.21[a]	4.23±0.30[a]	4.49±0.16[a]
0.5	1.04±0.07	1.99±0.12	3.03±0.32	3.84±0.59	5.16±0.61	6.20±0.96
3	0.94±0.14	1.31±0.18	2.21±0.15	3.56±0.08	4.81±0.01	5.60±0.10
6	1.04±0.19[a]	1.91±0.20[a]	2.54±0.20[a]	3.47±0.20	5.24±0.44[a]	6.75±0.45[a]
9	0.91±0.06	1.95±0.03	2.70±0.19	3.99±0.70	5.27±0.45	6.48±0.36
0.5–9 mM (mean)	0.98±0.03	1.76±0.16	2.62±0.17	3.71±0.12	5.12±0.11	6.25±0.24

The values shown are means±S.E.M. from two or three[a] independent flasks inoculated from different stock cultures. ND = not determined.

Table 2. The effect of glucose and glutamine concentration on the intracellular adenylate energy charge. The adenylate energy charge is (ATP + $^1/_2$ ADP)/(ATP + ADP + AMP). Hybridomas were inoculated into T-flasks (25 cm^2) containing 11 ml NB-SFM at varying glucose and glutamine concentrations. Intracellular nucleotides were extracted after 5 h of incubation at 37 °C. The initial EC value of stock cultures was 0.91±0.01 (n = 3)

Glutamine (mM)	Glucose (mM)					
	0	1	3	5	10	25
0	ND	ND	ND	0.85±0.05[a]	0.94±0.01[a]	0.92±0.01[a]
0.5	0.36±0.02	0.40±0.01	0.56±0.03	0.67±0.03	0.83±0.00	0.95±0.00
3	0.47±0.03	0.37±0.03	0.50±0.02	0.71±0.04	0.87±0.01	0.96±0.02
6	0.36±0.03[a]	0.35±0.05[a]	0.46±0.03[a]	0.58±0.04	0.80±0.03[a]	0.96±0.02[a]
9	0.35±0.06	0.34±0.02	0.46±0.02	0.58±0.05	0.79±0.00	0.95±0.00
0.5–9 mM (mean)	0.38±0.03	0.36±0.01	0.49±0.02	0.63±0.03	0.81±0.02	0.96±0.01

The values shown are means ±S.E.M. from two or three[a] independent flasks inoculated from different stock cultures. ND = not determined.

cultures containing glutamine at 0–9 mM and glucose at 0–25 mM (Table 2). The initial energy charge of the cells was 0.91. Following incubation in medium with a glucose concentration of 5 mM or lower, the energy charge decreased with values of 0.36–0.38 recorded at low glucose concentrations (0–1 mM). Only at 25 mM glucose was there a consistent increase in the energy charge which was recorded at 0.96 in the presence of glutamine (>0.5 mM). There was no significant increase in energy charge at the high level of glucose in the absence of glutamine. However, in the presence of 25 mM glucose there was a significant increase in the energy charge on the addition of 0.5 mM glutamine but no further increase at higher glutamine concentrations up to 9 mM.

However, it was observed that supplementation of the medium containing 5–10 mM glucose with a minimal level of glutamine caused a significant decrease (0.1–0.2 units) in the adenylate energy charge ($p < 0.005$). This can be explained by an increase in the total intracellular adenylate nucleotide concentration resulting from glutamine which is a precursor for purine biosynthesis.

A representative graph of the intracellular concentration of the adenylate nucleotides and energy charge as a function of glucose concentration in the medium is shown in Figure 1. The values were taken from a cultures in which the glutamine concentration was 0.5 mM. Similar graphs were obtained from cultures with glutamine concentrations of 3–9 mM (data not shown). Figure 1 shows that the intracellular ATP concentration and energy charge increased concomitantly with an increase in glucose concentration to a maximum of 6.2 nmol/10^6 cells and 0.95, respectively. However, the ADP concentration followed a bell-shaped curve in response to an increasing glucose level. A maximum intracellular ADP concentration of 1.0 nmol/10^6 cells was present in cells exposed to 3–5 mM glucose.

The intracellular AMP concentration was at its

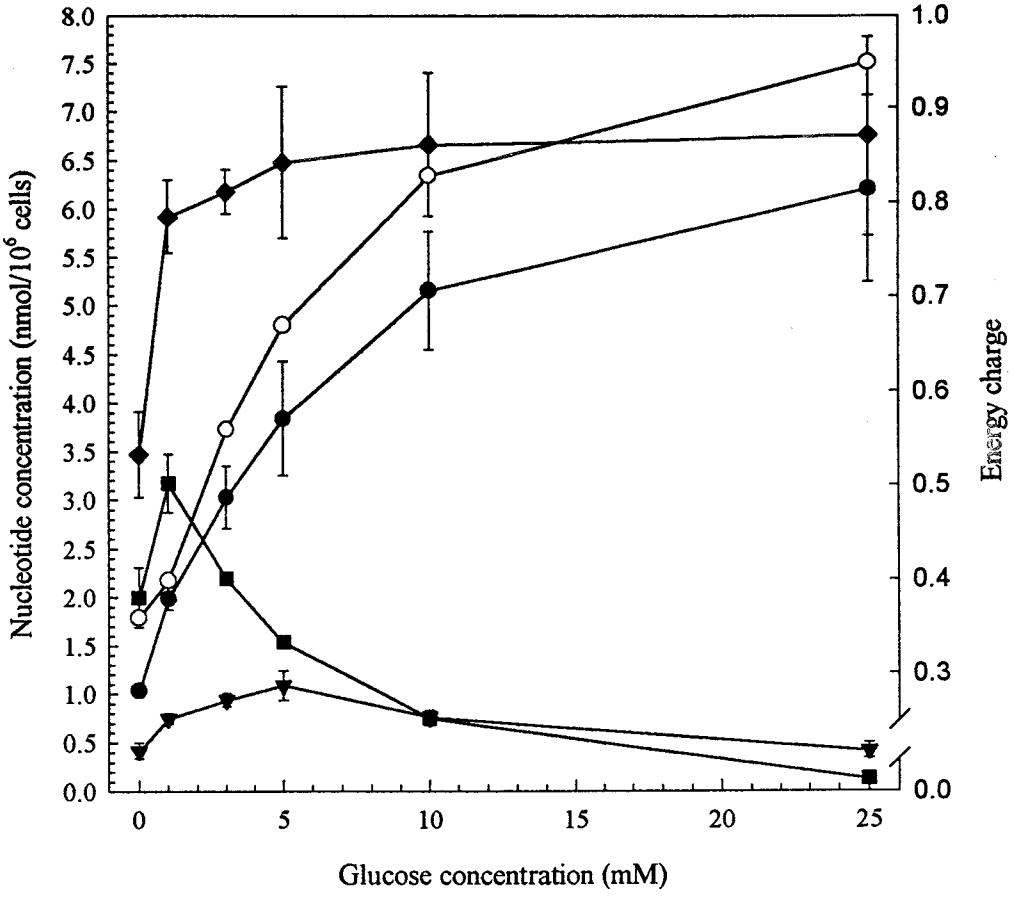

Figure 1. The effect of glucose on the intracellular adenylate nucleotide concentration. Hybridomas were inoculated into T-flasks (25 cm^2) containing 11 ml NB-SFM with varying glucose concentrations. All cultures contained 0.5 mM glutamine. Intracellular nucleotides were extracted after 5 h of incubation at 37 °C: ATP (●); ADP (▼); AMP (■); SA (♦) and energy charge (○). Values are the mean ±S.E.M. from two independent flasks inoculated from different stock cultures. Energy charge errors were less than 6% and not shown on the graph. Similar graphs were also obtained with cultures of hybridomas grown in glutamine concentrations of 3, 6 and 9 mM.

maximum value (3.17 nmol/10^6 cells) when cells were exposed to a low level of glucose (1 mM). At higher glucose concentrations there was a proportional decrease in the AMP pool down to a value below 0.1 nmol/10^6 cells. The total intracellular adenylate nucleotide concentration (SA–sum of adenylates) increased significantly in the presence of a minimum level of glucose and was relatively constant between 1–25 mM glucose (6.40 nmol/10^6 cells).

The effect of glucose and glutamine on non-adenylate nucleotides

The effect of glucose and glutamine on other nucleotide pools was also investigated (Figure 2, Table 3). An increase in extracellular glucose concentration resulted in the concomitant increase in UTP and GTP (Figure 2). In the absence of glucose, UTP and GTP concentrations were at their lowest values of 0.67 and 0.23 nmol/10^6 cells, respectively. Maximum concentrations of UTP (2.90 nmol/10^6 cells) and GTP (1.30 nmol/10^6 cells) were attained in the presence of 25 mM glucose. The UTP, GTP, UDP-GNac and NAD intracellular concentrations did not differ significantly in the range of 0.5–9 mM glutamine as determined by ANOVA (data not shown).

The intracellular UDP-GNac and NAD levels were constant regardless of the glucose concentration of the medium. The UDP-GNac concentration for cells exposed to 0–25 mM glucose was 1.48±0.06 nmol/10^6 cells while the NAD concentration was 0.65±0.04 nmol/10^6 cells. The intracellular CTP concentration was dependent on the glucose concentration of the medium (Table 3). A concentration of glucose

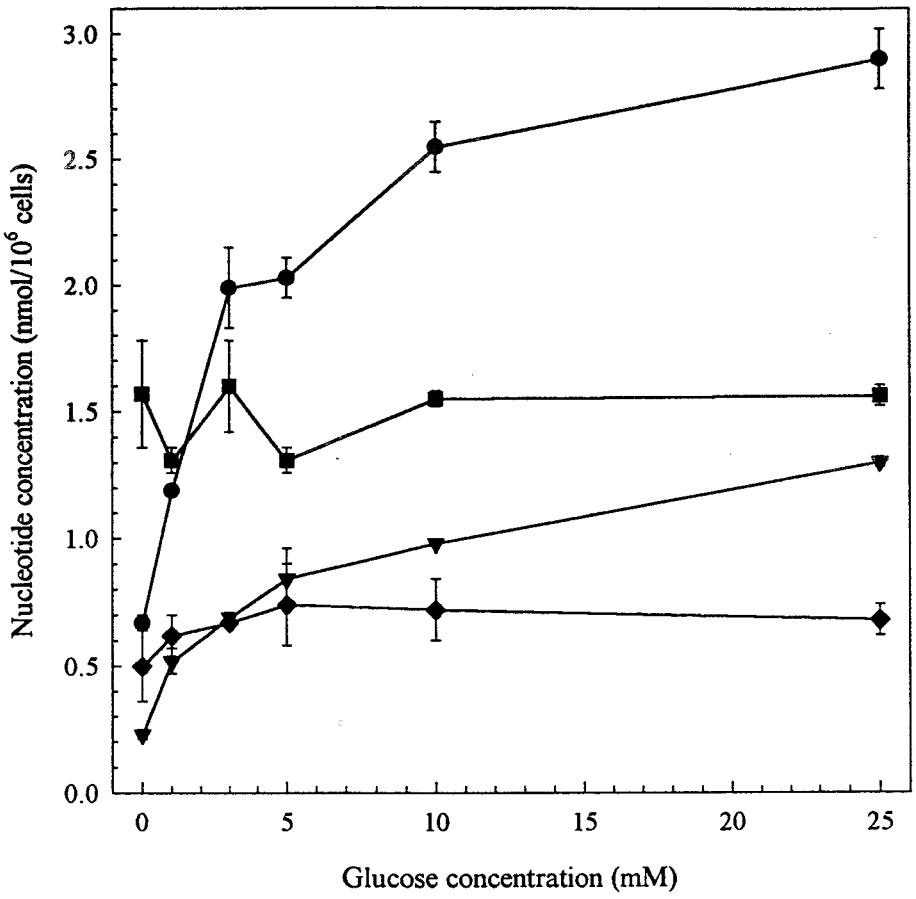

Figure 2. The effect of glucose on the intracellular concentrations of UTP, GTP, UDP-GNac and NAD. Hybridomas were inoculated into T-flasks (25 cm^2) containing 11 ml NB-SFM with varying glucose concentrations. All cultures contained 0.5 mM glutamine. Intracellular nucleotides were extracted after 5 h of incubation at 37 °C: UTP (●); GTP (▼); UDP-GNac (■); NAD (♦). Values are the mean ±S.E.M. from two independent flasks inoculated from different stock cultures. Similar graphs were also obtained with cultures of hybridomas grown in glutamine concentrations of 3, 6 and 9 mM.

of 5 mM or less resulted in an undetectable CTP level (<0.4 nmol/10^6 cells). Increasing the glucose concentration in the media to 10 or 25 mM resulted in the concomitant increase in the intracellular CTP level.

A summary of the effect of glutamine on the intracellular nucleotide pool is shown in Table 4. Exposing the cells to 0 mM glutamine and 25 mM glucose for 5 h did not change significantly the nucleotide levels in comparison to initial values at the time of inoculation. Supplementation with glutamine (0.5–9 mM) however caused a 65% increase in the total intracellular nucleotide pool. The greatest increase was for UTP (128%). The GTP, ATP, UDP-GNac and NAD pools increased by 65, 57, 47 and 45%, respectively. The total adenylate nucleotide pool increased by 44% in the presence of glutamine. In contrast, no significant changes were observed in the ADP and AMP pools. The CTP pool increased significantly on addition of glutamine irrespective of the glucose concentration ($p < 0.01$).

Similar results were obtained with respect to the effect of glutamine when cells were cultured in media containing 5 to 10 mM glucose (data not shown). There was no significant difference in the intracellular nucleotide concentrations within the glutamine concentration range 0.5–9 mM.

Effect of glucose and glutamine on the oxygen uptake rate

The effect of the extracellular concentration of glucose and glutamine on the cell specific oxygen uptake rate is shown in Table 5. Cultures exposed to 0 mM glutamine and 5, 10 or 25 mM glucose resulted in

Table 3. The effect of glucose and glutamine concentration on the intracellular CTP pool (nmol/10^6 cells). Hybridomas were inoculated into T-flasks (25 cm^2) containing 11 ml NB-SFM at varying glucose and glutamine concentrations. Intracellular nucleotides were extracted after 5 h of incubation at 37 °C. The initial CTP value of stock cultures was <0.4 nmol/10^6 cells (n = 3)

Glutamine (mM)	Glucose (mM)	
	10	25
0	<0.4	0.45±0.22[a]
0.5	0.56±0.23	0.92±0.09
3	0.73±0.08	0.74±0.43
6	0.75±0.26[a]	0.59±0.29[a]
9	1.12±0.03	0.90±0.12
0.5–9 mM (mean)	0.79±0.11	0.79±0.08

The values shown are means ±S.E.M. from two or three[a] independent flasks inoculated from different stock cultures. For cultures containing 0–5 mM glucose, the CTP concentration was below the limit of detection (<0.4 nmol/10^6 cells).

measured oxygen uptake rates of 0.12–0.15 μmol/h per 10^6 cells. Media containing the same level of glucose but with 0.5 mM glutamine resulted in the doubling of the oxygen uptake rate to 0.29 μmol/h per 10^6. However, a further increase in the glutamine concentration (>0.5 mM) did not result in a further increase in OUR.

The effect of glucose concentration on the specific oxygen uptake rate is shown in Figure 3. Supplementation of the medium with glucose caused an increase in the OUR up to a maximum value of 0.41 μmol/h per 10^6 cells at 0.1–1.0 mM glucose. This was significantly higher than the control culture containing no glucose ($p < 0.005$). The results shown in Figure 3 are for cultures with a glutamine concentration of 6 mM. However, there was no significant difference in the OUR within a glutamine concentration range of 0.5–10 mM ($p > 0.15$). Incubation of cells in a glucose concentration range of 5–25 mM resulted in an OUR that was not significantly different from that measured in the absence of glucose ($p > 0.25$).

Discussion

The purpose of the work described here was to determine the relative contribution of extracellular glucose and glutamine to the maintenance of intracellular nucleotide pools of a hybridoma in culture. Glucose and glutamine are the two major nutrients normally available for cell growth in standard culture medium. Both substrates are directly involved in intracellular nucleotide pool synthesis. In addition to supplying precursors for the *de novo* synthesis of nucleotides, glucose and glutamine provide energy by coupling catabolic or oxidative reactions to nucleotide phosphorylation (Henderson and Paterson, 1973). These nucleotide pools are fundamental to cellular metabolism and fluctuations in the nucleotide levels have been shown to lead to changes in cell behavior such as growth rate and differentiation (De Korte et al., 1987; Ryll and Wagner, 1992).

Analysis of nucleotide profiles shows that an increase in the glucose concentration of the culture medium from 0–25 mM was concomitant with an increase in the intracellular ATP pool. This suggests that the rate of glucose ATP production was dependent on the extracellular glucose level. This relationship was similar to that reported for cultured human myocytes (Frenes et al., 1992). Miller et al. (1989a) reported similar increases in intracellular ATP in a hybridoma but only up to 5 mM glucose. We found that for the CC9C10 hybridoma even at a high glucose concentration (5–25 mM) there was a concomitant increase in the intracellular ATP pool but without any change in the oxygen uptake rate. This suggests that the energy for increased ATP synthesis was provided by an anaerobic process such as glycolysis rather than by an oxidative pathway such as the TCA cycle. Measurements of the flux rates of the major pathways of glucose metabolism in hybridomas (including CC9C10) have shown that the glycolytic pathway to lactic acid contributes a very high proportion of the catabolic breakdown of glucose (Fitzpatrick et al., 1993; Petch and Butler, 1994).

Intracellular AMP levels decreased concomitantly with an increase in media glucose concentration from 1 to 25 mM. However, the AMP concentration as well as the total adenylate concentration of cells incubated in the absence of glucose was significantly lower than those incubated in a minimal level of glucose (1 mM). The total adenylate nucleotide pool of the CC9C10 cells was constant for glucose concentrations above 1 mM. The lower level of AMP in the absence of glucose may be explained by the probable deamination of AMP via adenylate deaminase, an enzyme that converts AMP to the non-adenylate nucleotide IMP (Atkinson, 1977).

$$\mathrm{AMP} + \mathrm{H_2O} \leftrightarrow \mathrm{IMP} + \mathrm{NH_3} \qquad (1)$$

In support of this explanation, previous studies have

Table 4. The effect of glutamine concentration on intracellular nucleotide pools (nmol/10^6 cells). Hybridomas were inoculated into T-flasks (25 cm^2) containing 11 ml NB-SFM supplemented with 25 mM glucose and varying concentrations of glutamine: 0, 0.5, 3, 6, 9 mM. Intracellular nucleotides were extracted after 5 h of incubation at 37 °C. 'Total' refers to the sum of all detectable nucleotides

	ATP	UTP	GTP	CTP	UDP-GNac	NAD	ADP	AMP	SA	Total
Initial values	3.98±0.59	1.26±0.18	0.73±0.15	0.40±0.10	1.03±0.11	0.47±0.07	0.57±0.11	0.14±0.08	4.68±0.78	8.72±1.32
0 mM glutamine[a]	4.49±0.16	1.56±0.44	0.81±0.28	0.45±0.22	0.72±0.30	0.44±0.12	0.50±0.10	0.15±0.02	4.65±0.72	9.46±1.36
0.5–9 mM glutamine[b]	6.25±0.24	2.88±0.12	1.21±0.03	0.79±0.08	1.52±0.09	0.68±0.04	0.41±0.05	0.07±0.02	6.75±0.29	14.43±0.63

[a] Values are the mean ±S.E.M. from 3 independent flasks inoculated from different stock cultures (n = 3).
[b] Values are the mean ±S.E.M. from cultures inoculated in 0.5, 3, 6 and 9 mM glutamine (n = 4).

Table 5. The effect of glucose and glutamine concentration on the specific oxygen uptake rate (μmol/h per 10^6 cells). The values shown are means ±S.E.M. from independent flasks (n = 2)

Glutamine (mM)	Glucose (mM)					
	0	1	3	5	10	25
0	ND	ND	0.15±0.02**	0.14±0.02**	0.12±0.01**	
0.5	0.29±0.01	0.42±0.04*	0.29±0.03	0.29±0.02	0.25±0.02[a]	
3	0.28±0.00[b]	0.43±0.04*	ND	ND	0.28±0.03	
6	0.27±0.02[a]	0.38±0.04*[a]	0.31±0.01	0.31±0.03	0.30±0.02[c]	
8	0.24±0.03	0.42±0.04*	ND	ND	ND	
10	0.20±0.05	0.41±0.02*	ND	ND	ND	

[a] (n = 4), [b](n = 3) or [c] (n = 12) inoculated from different stock cultures.
ND = not determined.
* $p < 0.005$ compared to 0 mM glucose cultures. ** $p < 0.007$ compared to 0.5–6 mM glutamine cultures. Statistical analysis was done by ANOVA.

shown that the production of IMP in ascites tumor cells is concomitant with a decrease in the adenylate pool and energy charge (McComb and Yushok, 1964; Lomax and Henderson, 1973). Furthermore, ATP is an allosteric inhibitor of adenylate deaminase (Ronca-Testoni and Ronca, 1974). Therefore, a decrease in ATP concentration in the absence of glucose could result in enhanced adenylate deaminase activity.

Analysis of the glucose-dependent ADP concentration resulted in a bell-shaped curve. Maximum ADP levels were obtained when cells were exposed to 3 and 5 mM glucose but the ADP levels were lower at high or low glucose concentrations (significance at $p < 0.1$). This curve is similar to that described by Atkinson (1977) who plotted the relative concentration of ADP as a function of energy charge assuming an adenylate kinase reaction equilibrium. Adenylate kinase transfers activated phosphate groups reversibly between adenylate nucleotides (Equation 2).

$$\text{AMP} + \text{ATP} \leftrightarrow 2\ \text{ADP} \qquad (2)$$

The dependency of all three adenylate nucleotides on the extracellular glucose concentration results in a gradual increase in the adenylate energy charge with an increase in glucose concentration from a low value of 0.38 in the absence of glucose to 0.97 at 25 mM glucose. The total adenylate nucleotide pool of cells exposed to 1–25 mM glucose was constant, a fact that suggests that glucose does not stimulate the *de novo* synthesis of nucleotides.

An increase in glucose concentration was also related to a concomitant increase in the GTP, UTP and CTP pools similar to the increase observed in the ATP pool. The increase in these three nucleotide pools is probably due to the acquisition of the terminal phosphate group from ATP in reactions catalyzed by nucleoside diphosphokinases (Henderson and Paterson, 1973).

The glucose concentration in the media did not affect the UDP-GNac or NAD pools. Although glucose is a precursor of the UDP-GNac pool (Ryll et al., 1994), it may be that UDP-GNac synthesis is

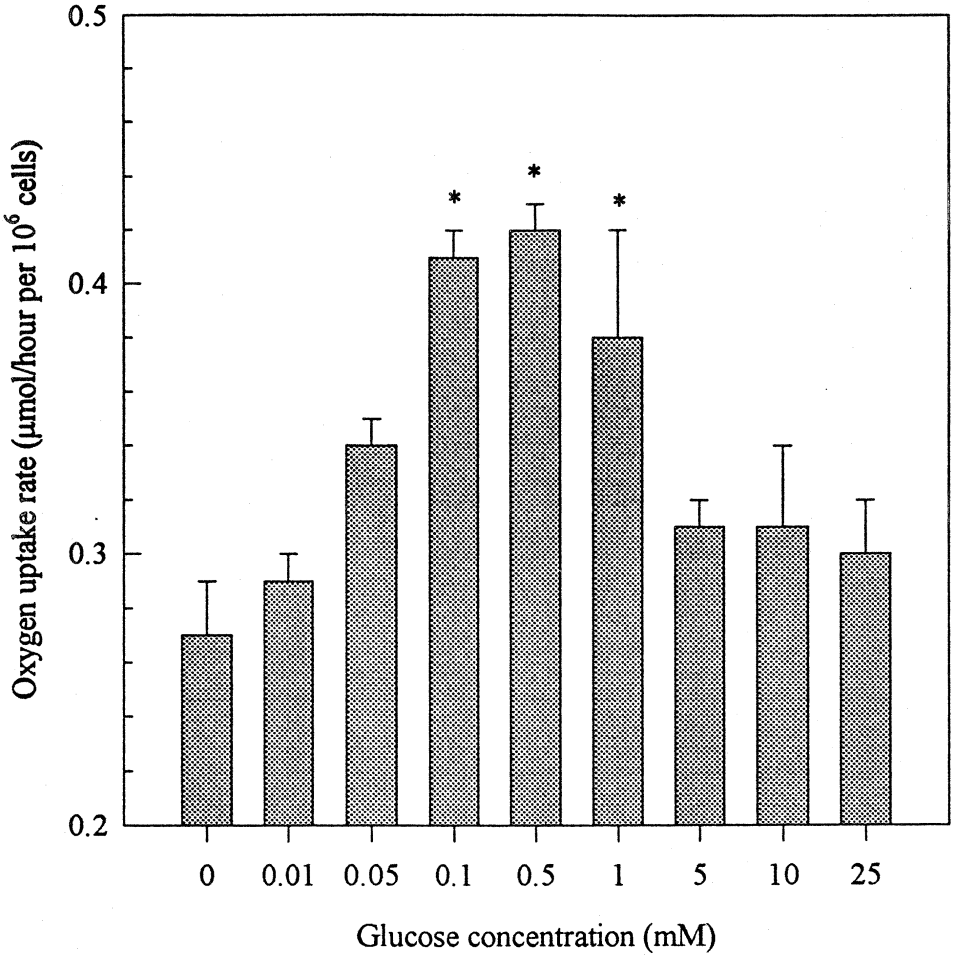

Figure 3. The effect of glucose concentration on the rate of cell respiration. Hybridomas were inoculated into T-flasks (25 cm^2) containing 11 ml NB-SFM with varying glucose concentrations. All cultures contained 6 mM glutamine. The oxygen uptake rates shown are means±S.E.M. from two independent flasks inoculated from different stock cultures except for 0 mM glucose (n = 4) and 25 mM glucose (n = 12). $* p < 0.05$ compared to base line values at 0 mM glucose.

more dependent on another precursor such as ammonia. In contrast, it was observed that the addition of glutamine to the medium did result in an increase in the UDP-GNac pool which may be explained by an increased production of ammonia. This result is important in bioprocesses because variability in protein glycosylation has been shown to be related to changes in the intracellular UDP-GNac pool (Grammatikos et al., 1998).

Glucose is not a direct precursor for the synthesis of NAD and therefore it is not surprising that there was no apparent effect of glucose concentration on the intracellular level of this co-enzyme. The oxidative state of NAD may change during glycolysis but for lactate formation there would be no net change and the total concentration of the co-enzyme would remain the same (Lehninger, 1982).

The cells responded differently to a variation in the concentration of extracellular glutamine. Glutamine is known to be a precursor for *de novo* synthesis of intracellular nucleotides (Engstrom and Zetterberg, 1984) and a minimal level is required to stimulate cell growth (Glacken, 1988; Christie and Butler, 1999). The addition of a minimal level of glutamine (0.5 mM) to the medium of the CC9C10 hybridomas caused a 45–128% increase in the total adenylate nucleotide pool and all nucleotide triphosphates, NAD and UDP-GNac levels compared to cultures exposed to 0 mM glutamine. The presence of a minimal level of glutamine also caused a 0.1–0.2 unit decrease in the energy

charge level in cultures exposed to 5–10 mM glucose. This decrease in the energy charge is explained by an increase in the *de novo* synthesis of the total adenylate pool especially ADP and AMP. Energy charge values also decreased in the presence of glutamine in isolated mesenteric lymphocytes (Ardawi and Newsholme, 1983).

In contrast to the above, increases in glutamine concentration from 0.5–9 mM did not significantly alter nucleotide pools, the energy charge nor the oxygen uptake rate. This indicates that a limitation in glutamine utilization may exist. It has been proposed that at a glutamine concentration higher than 2 mM, the transport of this amino acid is the limiting factor in its utilization (Fitzpatrick et al., 1993). Another possible rate limiting step in glutamine metabolism may be the reduced activity of enzymes of the glutaminolysis pathway (Glacken, 1988; Sri-Pathmanathan et al., 1990). Enzymes function as regulators of glutaminolysis and may be slow in responding to changes in the glutamine concentration (Neerman and Wagner, 1996).

The relative contribution of glucose and glutamine to the energy status of the cells was determined from the increase in intracellular ATP concentration in cultures at varying levels of the two substrates. The estimate was based on cultures in which the ATP level increased from the original cell inoculum. This occurred in our experiments in cultures containing 10–25 mM glucose and 0.5–9 mM glutamine. From these it was estimated that the metabolism of glutamine provided the metabolism for the production of between 17–28% of the intracellular ATP pool while glucose supplied the remainder.

A previous estimate from our laboratory of the relative contribution of glucose and glutamine metabolism to the intracellular energy state of CC9C10 cells was based on metabolic flux analysis using radioactive precursors (Petch and Butler, 1994). In that report it was estimated that glutamine contributed close to 41% of the total ATP production in cultures containing 20 mM glucose and 2 mM glutamine. That estimate was based on an assumption of the stoichiometric relationship between the molar rate of glutamine metabolism via glutaminolysis and ATP production (9 ATP/gln metabolised). However, that assumption may well over-estimate the contribution of glutaminolysis to energy metabolism if oxidative phosphorylation is uncoupled from the catabolism of glutamine. In the data presented in this paper it was shown that there was no increase in the oxygen utilization rate of the cells when the glutamine concentration was increased above 0.5 mM. That suggests that oxidative phosphorylation may well be uncoupled from glutaminolysis at the higher glutamine concentrations.

The oxygen uptake rate of CC9C10 hybridomas in the presence of 5–25 mM glucose and 0.5–6 mM glutamine was approximately 0.29 μmol/h per 10^6 cells. This value is similar to the value reported previously for the same cell line (Jan et al., 1997). Removal of glutamine from the media reduced the oxygen uptake rate by 50% to approximately 0.14 μmol/h per 10^6 cells. This may be explained by not only a reduction in glutaminolysis but also a reduction in TCA cycle intermediates which would reduce the oxidative metabolism of all substrates including glucose. Although, the contribution of glucose and glutamine to energy metabolism is emphasized in our results, other endogenous fuels such as lipids and amino acids may also be metabolised oxidatively (Guppy et al., 1997).

Maximum oxygen uptake rates were observed when cells were exposed to 0.1–1 mM glucose in the presence of glutamine. Increasing the extracellular glucose concentration above 1 mM resulted in a 30% decrease in the respiration rate. This inhibition of respiration at high glucose levels is a well-established phenomenon commonly referred to as the Crabtree effect (Dell'Antone, 1994). A similar reduction in respiration rates has been shown in cultures of swine testicular cells and fibroblasts at glucose levels above 3 mM (Glacken, 1988; Wohlpart et al., 1990). This also explains the low levels of oxidative metabolism generally observed in standard batch cultures of hybridomas (Fitzpatrick et al., 1993; Petch and Butler, 1994).

We conclude from this experimental data that the major metabolic role of glucose at the concentration range prevalent in cell culture (10–25 mM) is to provide a mechanism for the phosphorylation of adenylate nucleotides via aerobic glycolysis. On the other hand the minimal level of glutamine (0.5 mM) is necessary to ensure the biosynthesis of the adenylate nucleotide pool and to provide intermediates in the TCA cycle. The requirement for this minimal level of glutamine is supported by growth experiments with other cell lines (Christie and Butler, 1999).

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