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Glucose and Glutamine Metabolism of a Murine B-Lymphocyte Hybridoma Grown in Batch Culture

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ABSTRACT

The energy metabolism of a mammalian cell line grown in vitro was analyzed by substrate consumption rates and metabolic flux measurements. The data allowed the determination of the relative importance of the pathways of glucose and glutamine metabolism to the energy requirements of the cell. Changes in the substrate concentrations during culture contributed to the changing catalytic activities of key enzymes, which were determined.

1. A murine B-lymphocyte hybridoma (PQXB1/2) was grown in batch culture to a maximum cell density of $1\text{–}2 \times 10^6$ cells/mL in 3–4 d. The intracellular protein content showed a maximum value during the exponential growth phase of 0.55 mg/10^6 cells. Glutamine was completely depleted, but glucose only partially depleted to 50% of its original concentration when the cells reached a stationary phase following exponential growth.
2. The specific rates of glutamine and glucose utilization varied during culture and showed maximal values at the midexponential phase of 2.4 nmol/min/10^6 cells and 4.3 nmol/min/10^6 cells, respectively.

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3. A high proportion of glucose (96%) was metabolized by glycolysis, but only limited amounts by the pentose phosphate pathway (3.3%) and TCA cycle (0.21%).
4. The maximum catalytic activity of hexokinase approximates to the measured flux of glycolysis and is suggested as a rate-limiting step. In the stationary phase, the hexokinase activity reduced to 11% of its original value and may explain the reduced glucose utilization at this stage.
5. The maximal activities of two TCA cycle enzymes were well above the measured metabolic flux and are unlikely to pose regulatory barriers. However, the activity of pyruvate dehydrogenase was undetectable by spectrophotometric assay and explains the low level of flux of glycolytic metabolites into the TCA cycle.
6. A significant proportion of the glutamine (36%) utilized by the cells was completely oxidized to CO_2.
7. The measured rate of glutamine transport into the cells approximated to the metabolic flux and is suggested as a rate-limiting step.
8. Glutamine metabolism is likely to occur via glutaminase and amino transaminase, which have significantly higher activities than glutamate dehydrogenase.
9. The calculated potential ATP production suggests that, overall, glutamine is the major contributor of cellular energy. However, at the midexponential phase, the energy contribution from the catabolism of the two substrates was finely balanced—glutamine (55%) and glucose (45%).

Index Entries: Hybridoma; glucose; glutamine; enzymes; metabolic flux; energy metabolism; glycolysis; glutaminolysis.

INTRODUCTION

The importance of characterizing the growth and metabolism of B-lymphocyte hybridomas in batch culture has increased with the development of large-scale processes for monoclonal antibody production (*1,2*). The maximization of production rates in a batch process requires an understanding of the changing state and metabolism of the cells. Such an understanding can lead to the development of control strategies that allow high growth yields from the available nutrients and minimal byproduct formation (*3*).

Glucose and glutamine are the major carbon sources utilized by hybridomas in culture (*4*), both substrates being provided at relatively high concentration (glucose: 5–25 m*M*; glutamine: 1–5 m*M*). Glucose catabolism has been reported that very little glucose carbon enters the tricarboxylic acid cycle in cultured cells (*5,6*). Glucose also provides precursors for ribose formation through the pentose phosphate pathway, which is particularly important for nucleic acid synthesis during cell growth (*7,8*). Glutamine

can undergo complete oxidation to carbon dioxide or partial breakdown to three or four carbon metabolites. The latter process has been termed glutaminolysis (*9*). The catabolism of glutamine can provide a substantial proportion of the cellular energy requirement (*10*), particularly in mitogenically stimulated lymphocytes (*11*). Glutamine is also required as a precursor for nucleic acid synthesis (*12*).

Glucose and glutamine have also been recognized as the major carbon substrates for lymphocytes in vivo. Rates of utilization of both glucose and glutamine are enhanced following an immunological challenge of lymphocytes (*13*). They provide the energy requirements of the cells and precursors necessary for macromolecule synthesis during cell growth. However, the high rate of flux of these substrates through glycolysis and glutaminolysis has been attributed to the need for a precise and sensitive control system that can supply the biosynthetic pathways as required for cell proliferation (*14*). This metabolic control system ensures an efficient response of the immune system in vivo.

During batch culture, the metabolism of cells changes as substrates are utilized and metabolic byproducts are released into the medium. This gives rise to a changing intracellular profile of enzymic activities, which can affect the state of the cells with respect to growth and productivity. Maximum catalytic activities of certain enzymes provide a quantitative indication of maximal flux through metabolic pathways (*15*). Enzymic activities that are similar to the flux suggest roles as flux indicators or regulators (*13,16–18*).

The purpose of the work reported here was to determine the pattern of glucose and glutamine metabolism during the growth of a B-lymphocyte hybridoma in batch culture. Metabolic flux analysis provided information regarding the metabolic pathways followed by these substrates. This was related to the varying specific consumption rates of the two substrates to determine their relative importance in providing the energy requirements of the cells. Analysis of the maximum catalytic activities of some of the key enzymes of glucose and glutamine metabolism is reported to indicate the rates of maximal flux and suggest rate-limiting steps.

MATERIALS AND METHODS

Cell Line

A murine B-lymphocyte hybridoma (PQXB1/2) was obtained from the European Collection of Animal Cell Cultures (ECACC, PHLS, Salisbury, UK). The cells produced a monoclonal antibody against paraquat (*19*).

Growth Medium

Cells were grown in RPMI 1640 medium containing 10 m*M* glucose, 2 m*M* glutamine, and 25 m*M* sodium bicarbonate, supplemented with 10% (v/v) heat-treated horse serum. Medium and serum were purchased from Imperial Laboratories (Hampshire, UK).

Chemicals

Chemical reagents were of the highest grade and obtained from Sigma Chemical Company, Dorset, UK. Radioactive material was obtained from Amersham, Bucks, UK.

Cultures

Cells are routinely grown in 25 cm^2 T-flasks. Experimental cultures (250 mL) were maintained in spinner flasks stirred at 30 rpm in a 37°C incubator containing an atmosphere of 10% CO_2. Viable cell numbers were determined by counting with a Neubauer hemocytometer using the trypan blue exclusion method (*20*).

ELISA

Culture supernatants were assayed for antibody by a specific ELISA that involved the binding of the Mab to a paraquat-BSA conjugate.

Media Deproteinization

Media samples were deproteinized with perchloric acid (final concentration 2% w/v). Acidified extracts were incubated on ice for 15 min before centrifugation at $8000 \times g$ for 5 min. Supernatants were neutralized with 2*M* KOH containing 0.5*M* triethanolamine and were stored at −20°C until assay for glucose or lactate.

Assay of Medium

1. Glutamine was determined from ammonia released by the action of glutaminase (*21*).
2. Ammonia was determined by the method of Fawcett and Scott (22).
3. Glucose was determined colorimetrically in deproteinized media samples using the *o*-toluidine reagent (Sigma kit 635).
4. Lactate was determined in deproteinized media samples enzymatically by the reduction of NAD in the presence of lactate dehydrogenase (23).

Cell Extracts for Protein and Enzyme Analysis

Cells were harvested by centrifugation at 500×*g* for 5 min at room temperature. The cell pellet was washed twice with 10 mL Krebs-Henseleit bicarbonate buffer, pH 7.4, before resuspension in 5–10 vol of the appropriate enzyme extraction buffer at 4°C. The cells were lysed by two freeze-thaw cycles. The lysates were centrifuged at 8000×*g* for 5 min, and the resulting supernatants were used for assay. A different extraction buffer was used for each enzyme as cited by Ardawi and Newsholme (*16*). All extraction buffers contained 0.05% (v/v) Triton X-100.

Protein Determination

Protein concentrations of cell extracts were determined colorimetrically by the Bradford reagent (*24*).

Enzyme Analysis

All enzyme activities were measured at 25°C by spectrophotometric assays using a Cecil (Model 292) spectrophotometer. Enzyme activities were determined from the rate of change of absorbance at 340 nm. Each assay was validated by showing a linear relationship between the addition of enzyme and spectrophotometric response.

The assays were based on methods previously described:

1. Enzymes of glucose metabolism: hexokinase (HK) (*25*); 6-phosphofructokinase-1 (PFK) (*26*); pyruvate kinase (PK) (*27*); lactate dehydrogenase (LDH) (*28*); pyruvate dehydrogenase (PDH) (*29*).
2. Enzymes of glutamine metabolism: phosphate-activated glutaminase (PAG) (*30*); aspartate transaminase (AT) (*31*); glutamate dehydrogenase (GDH) (*32*); glutamine synthetase (GS) (*33*).
3. Enzymes of the TCA cycle: citrate synthetase (CS) (*34*); 2-oxoglutarate dehydrogenase (OGDH) (*15*).

Radioactivity

All radioactive samples were counted in Ecoscint A using an LKB Rackbeta II liquid scintillation counter.

Membrane Transport

The rate of transport of substrates through membranes is generally measured by the use of analogs that are not metabolized. In the case of glucose, a radioactively labeled analog (2-deoxy-D-[-1-^{3}H] glucose) was used (*35*). Glutamine transport was measured as previously described (*36,37*).

Cells (1–2×10^7 cells/mL) were suspended in growth medium containing 2-deoxy-D-[-1-^{3}H] glucose (0.1 μCi). Replicate suspensions (800 μL) were incubated at 37°C under a 10% CO_2 atmosphere. At regular intervals up to 10 min, a sample (500 μL) was taken and centrifuged (8000×*g*, 25 s) through a layer of 1:1 (v/v) mixture (300 μL) of Dow Corning 550 silicone oil and dinonyl phthalate into 20% (w/v) perchloric acid (100 μL). Radioactive counts were made of the cell extract in the acid layer.

In parallel experiments, cells were incubated with ^{3}H-inulin (0.1 μCi) to act as a marker of the extracellular space and with ^3H_2O (0.1 μCi) to measure total volume. Intracellular volume was determined as the difference in volume available to ^3H_2O and ^{3}H-inulin (*37*). Sixty-five percent of the total pellet volume was determined as extracellular space.

Metabolic Flux

1. Glutamine oxidation was measured by the rate of $^{14}CO_2$ release from L-[U-^{14}C]-glutamine (*38*).
2. TCA cycle flux from glucose catabolism was measured by the rate of $^{14}CO_2$ release from 6-^{14}C-glucose (*39*).
3. Pentose phosphate pathway flux was measured by the difference in the rate of $^{14}CO_2$ from 1-^{14}C-glucose and 6-^{14}C-glucose (*39*).

   The protocol for measurement of CO_2 evolution was as follows: Cells were suspended in growth medium at a concentration of 5×10^6 cells/mL. Aliquots (1 mL) were dispensed into glass scintillation vials (15 mL), which were stoppered with a Subaseal containing a Konte center well with a filter paper wick. A radioactive substrate—L-[U-^{14}C]-glutamine (1 μCi), 1-^{14}C-glucose (0.45 μCi), or 6-^{14}C-glucose (0.45 μCi)—was added to each cell suspension. The vials were shaken at 80 rpm at 37°C for a period up to 4 h. At each time-point, a 1:1 (v/v) mixture (300 μL) of phenylethylamine and methanol was injected into the center well to trap $^{14}CO_2$, and 10% (w/v) trichloroacetic acid (300 μl) was injected into the cell suspension. The vials were incubated for a further 1 h to ensure complete absorption of $^{14}CO_2$ into the filter paper, which was counted for radioactivity.
4. Glycolytic flux was determined from the rate of release of ^3H_2O from metabolized D-[3-^{3}H] glucose (*40*). Cells (3–4×10^6) were incubated in growth medium (1 mL) containing D-[3-^{3}H]-glucose (1 μCi). At each time-point up to 4 h, 300 μL of 10% (w/v) trichloroacetic acid were added to the cell suspension. The samples were neutralized with KOH (2*M*) containing triethanolamine (0.5*M*) and centrifuged at 7500×*g* to remove any precipitate. The supernatant was applied to an Amberlite CG400 column (1 mL) in the borate form, which was washed with 3

mL distilled water to collect the ^3H_2O from the glucose and glycolytic metabolites that attached to the column. The eluent was counted for radioactivity.

Radiolabeling of Cell Proteins

Incorporation of L-[U-^{14}C]-glutamine into total protein was determined by precipitation of a cell suspension (50 μL) with 50 μL of cold 10% (w/v) trichloroacetic acid containing glutamine (10 m*M*). The centrifuged pellets were washed three times with cold 5% (w/v) trichloroacetic acid containing glutamine (5 m*M*) before radioactive counting.

Potential ATP Production

The potential ATP production was calculated from the consumption rate of each substrate, the distribution of metabolites through alternative pathways, and theoretical maximum ATP yields. The theoretical yields for glucose utilization were based on 36 ATP mol/mol glucose for TCA cycle and 2 ATP mol/mol for lactate formation. The yields for ATP formation from glutamine were based on 27 ATP mol/mol for complete oxidation and 9 ATP mol/mol for glutaminolysis, which would include the formation of aspartate, lactate, or alanine (*41*).

RESULTS

Cell Growth, Intracellular Protein, and Antibody Production

The PQXB1/2 cells grew at a maximum rate in batch culture for 3 d with a doubling time of 16.7 h reaching a maximum cell density of 1.4×10^6 viable cells/mL (Fig. 1). A cell viability of > 90% was maintained throughout the culture. The intracellular protein content of the cells varied from a maximum of 0.55 mg/10^6 cells after the first day of growth to a minimum of 0.17 mg/10^6 cells in the stationary phase.

The antibody production of these cells varied from a maximum culture concentration of 100 μg/mL for early passage cells to 40 μg/mL after 35 passages from the original stock culture. The PQXB1/2 cultures reported here produced 30–40 μg/mL antibody. The effect of passage number on antibody productivity has been previously reported (*42*).

Substrate Utilization and Byproduct Formation

The consumption of glucose and glutamine by the cells in batch culture is shown (Fig. 2). Glutamine was completely depleted from the medium

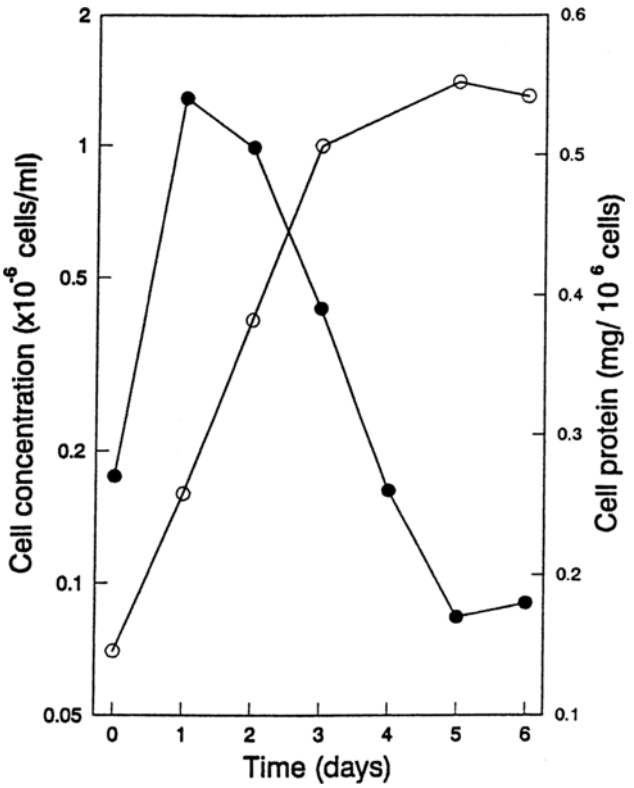

Fig. 1. Intracellular protein concentration during growth of PQXB1/2 cells. PQXB1/2 hybridomas were grown in RPMI 1640 supplemented with 10% horse serum in 250-mL spinner flasks with a stirring speed of 30 rpm. At daily intervals, samples (1 mL) were taken for the determination of cell concentration (○) and intracellular protein (●).

in 5 d, which corresponded to the stationary phase of the culture. Glucose was reduced to 50% of its original concentration during this period.

The specific substrate utilization rates vary during culture (Fig. 3). The rate of glutamine utilization increased to a maximum value of 2.4 nmol/min/10^6 cells at day 2. This corresponded to the midexponential phase of the culture and at a point when the glutamine concentration was still relatively high in the medium. This was followed a day later by the maximum glucose consumption rate of 4.2 nmol/min/10^6 cells.

The concentrations of lactate and ammonia gradually increased in the media, largely as a result of the metabolism of glucose and glutamine. The product yields expressed as metabolic coefficients, lactate/glucose (mol/mol) and ammonia/glutamine (mol/mol), were calculated from the cumulative substrate consumption and byproduct formation. The calculated values after 4 d of cell growth were 2.05 for lactate/glucose and 0.64 for ammonia/glutamine. If direct conversion is assumed, then this would

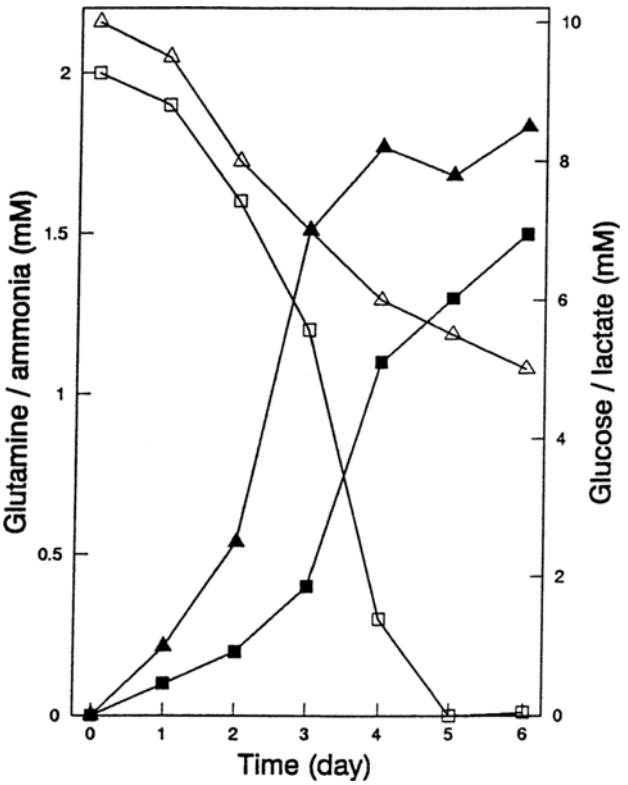

Fig. 2. Substrate utilization and byproduct formation by PQXB1/2 hybridomas. The supernatants of samples (1 mL) taken from PQXB1/2 hybridoma cultures (250 mL) were analyzed for glutamine (□), glucose (△), ammonia (■), and lactate (▲) at daily intervals. Deproteinized samples were used for glucose and lactate analysis as described in Materials and Methods.

indicate that most of the glucose is metabolized to lactate, and over half of the nitrogen of glutamine is released as ammonia. Previous calculations of these coefficients have shown that they are highly dependent on the medium composition (*43*).

Membrane Transport of the Carbon Substrates

The rates of uptake of radiolabeled substrates were measured over relatively short time periods into cells taken at midexponential growth phase (Fig. 4). A glucose analog (2-deoxy-D-[-1-^{3}H] glucose) was used to determine the rate of glucose transport independently from any effects of cellular metabolism. In the case of glutamine, a suitable radioactive analog was not available, but in a preliminary experiment, the transport of L-[U-^{14}C] glutamine was measured in the presence of the metabolic inhibitor,

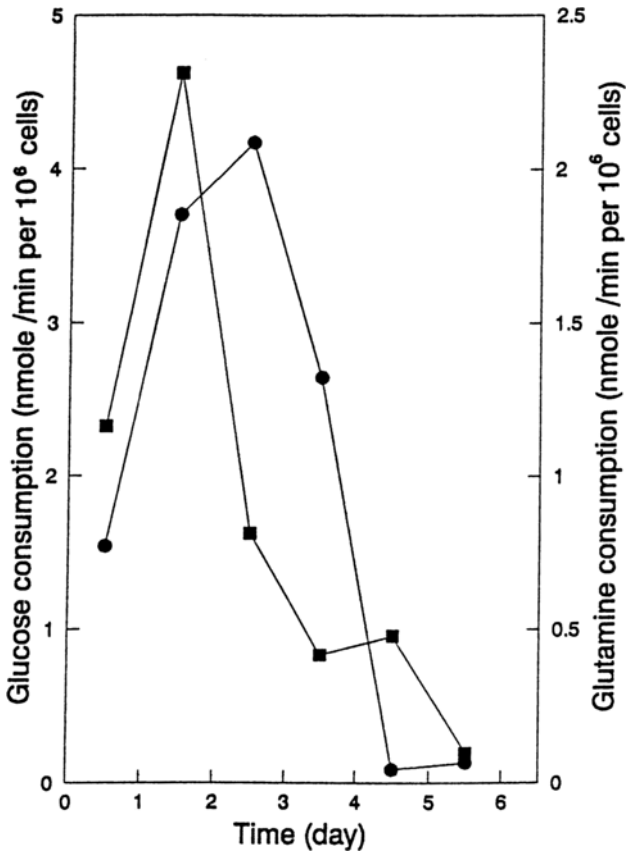

Fig. 3. Rates of substrate utilization during growth of PQXB1/2 hybridomas. The rates of glucose (●) and glutamine (■) utilization by PQXB1/2 hybridomas were determined on a daily basis from the analysis of cell and substrate concentrations as shown in Figs. 1 and 2.

aminooxyacetate. From this preliminary experiment, it was shown that measured utilization rates were not affected by the inhibitor (*36*).

Figure 4 shows the results of the measurement of glucose and glutamine transport into the cells taken from the midexponential phase of growth. The transport rates were determined from the initial linear portion of each curve. For glucose transport, the rate was 16 nmol/min/10^6 cells, and for glutamine transport, the rate was 1.46 nmol/min/10^6 cells. In the case of glutamine, transport was also measured at the stationary phase of culture when a reduction to a value of 0.73 nmol/min/10^6 cells occurred.

Metabolic Flux

Glucose Metabolism

The flux of glucose through three pathways was measured by incubation of cells with specifically radiolabeled glucose molecules. The release

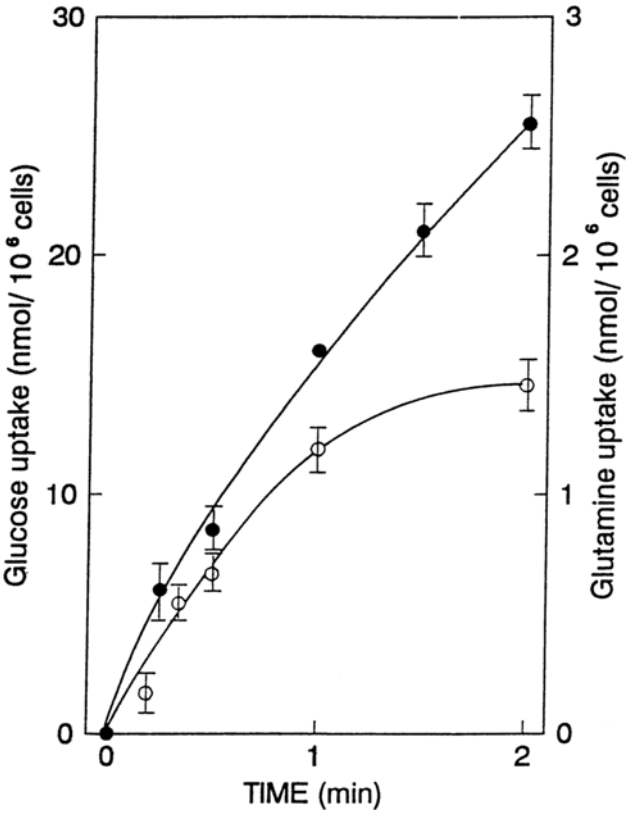

Fig. 4. Membrane transport of ^{14}C-substrates into PQXB1/2 cells. The rate of transport of the glucose analog, 2-deoxy-D-[-1-^{3}H]-glucose (●) and L-[U-^{14}C]-glutamine (○) into PQXB1/2 hybridomas was determined in short-term experiments as described in Materials and Methods. The cells were obtained from the midexponential phase of culture and incubated in normal growth medium supplemented with the radioactive substrates during the course of the experiment. Each point represents the mean of three independent determinations. The error bars indicate standard errors.

of $^{3}H_2O$ from D-[3-^{3}H]-glucose measures the flux of glucose metabolites through the aldolase and triose phosphate isomerase reactions, which are committed steps of the glycolytic pathway. The measured rate of release of $^{3}H_2O$ following incubation with hybridomas taken from midexponential phase (48 h) of culture was constant for 2 h at 3.75 nmol/min/10^6 cells (Fig. 5). This represents 96% of the total glucose metabolized via the three catabolic pathways determined in these experiments.

The flux of glucose metabolites through the TCA cycle was measured from the rate of release of $^{14}CO_2$ from 6-^{14}C-glucose, which was constant for 2 h of incubation with cells taken from midexponential phase (48 h). The rate was 0.008 nmol/min/10^6 cells, which represented 0.21% of glucose

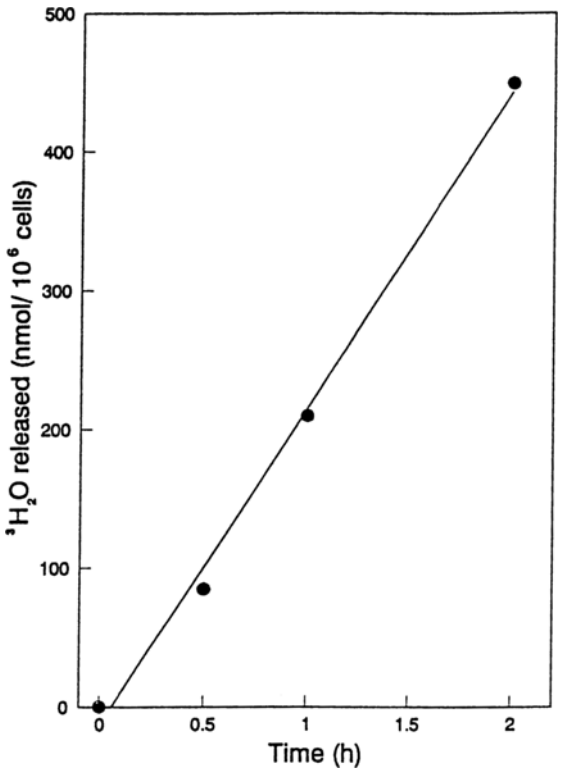

Fig. 5. The rate of release of ^3H_2O from the metabolism of D-[3-^{3}H]-glucose. The rate of glycolytic flux in the PQXB1/2 hybridomas was measured by the rate of release of ^3H_2O from cells ($3–4 \times 10^6$) incubated in normal growth medium (1 mL) containing D-[3-^{3}H]-glucose (1 μCi) as described in Materials and Methods. The cells were taken after 48 h in batch culture. Each time-point represents the mean of three independent determinations. In each case, the standard error was below 20% of the mean value.

metabolized by the three catabolic pathways (Fig 6). Flux through the oxidative branch of the pentose phosphate pathway was measured by the difference in rates of $^{14}CO_2$ release from 1-^{14}C-glucose and 6-^{14}C-glucose at 0.129 nmol/min/10^6 cells, representing 3.3% of the glucose metabolized.

Glutamine Metabolism

The rate of release of $^{14}CO_2$ from L-[U-^{14}C]-glutamine was measured as an indicator of glutamine oxidation (Fig. 7). The cells that were taken from midexponential phase of culture oxidized glutamine to CO_2 at a rate of 0.38 nmol/min/10^6 cells. This rate was linear for 4 h and represents complete oxidation of 36% of the glutamine incorporated into the cell.

The glutamine incorporated into total protein was determined by radioactive counting of the acid precipitable material during incubations up to 5 h. The incorporation rate was linear over this time period at 0.067 nmol

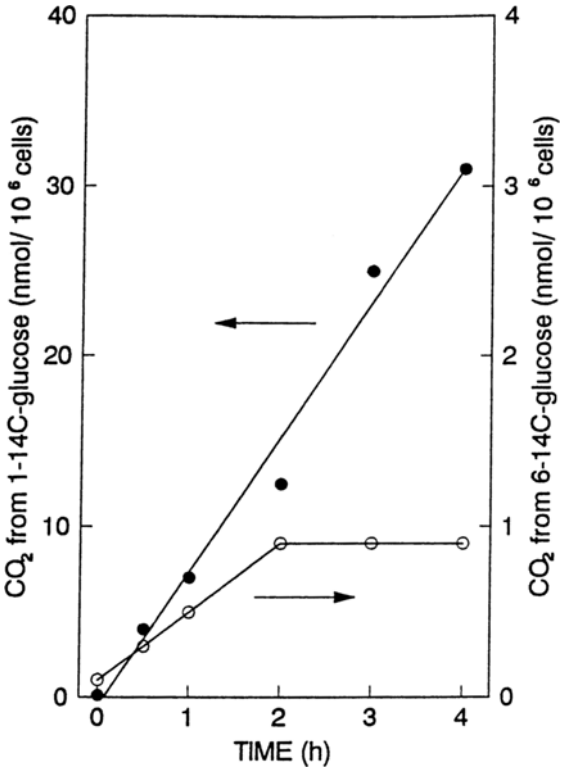

Fig. 6. The rate of $^{14}CO_2$ release from the metabolism of [1-^{14}C]- and [6-^{14}C]-glucose. The rate of $^{14}CO_2$ release was measured from PQXB1/2 hybridomas (5×10^6/mL) incubated in normal growth medium (1 mL) containing 0.45 μCi of [1-^{14}C]-glucose (●) or [6-^{14}C]-glucose (○) as described in Materials and Methods. The cells were taken after 48 h in batch culture. Each time-point represents the mean of three independent determinations. The standard errors were below 15% for determinations with [1-^{14}C]-glucose and below 20% for determinations with [6-^{14}C]-glucose.

glutamine/min/10^6 cells, which represents 6.3% of the glutamine incorporated into the cell. The flux rates determined for glucose and glutamine metabolism and membrane transport are summarized in Table 1. Some of the data for glutamine metabolism in these cells were previously reported (*36*).

Enzyme Analysis

The maximal catalytic activities were determined for a number of enzymes selected from the pathways of glycolysis, glutaminolysis, and the TCA cycle. Three time-points were chosen for analysis of the cells in batch culture—days 2, 4, and 7—representing early-, late exponential, and stationary phases (Table 2).

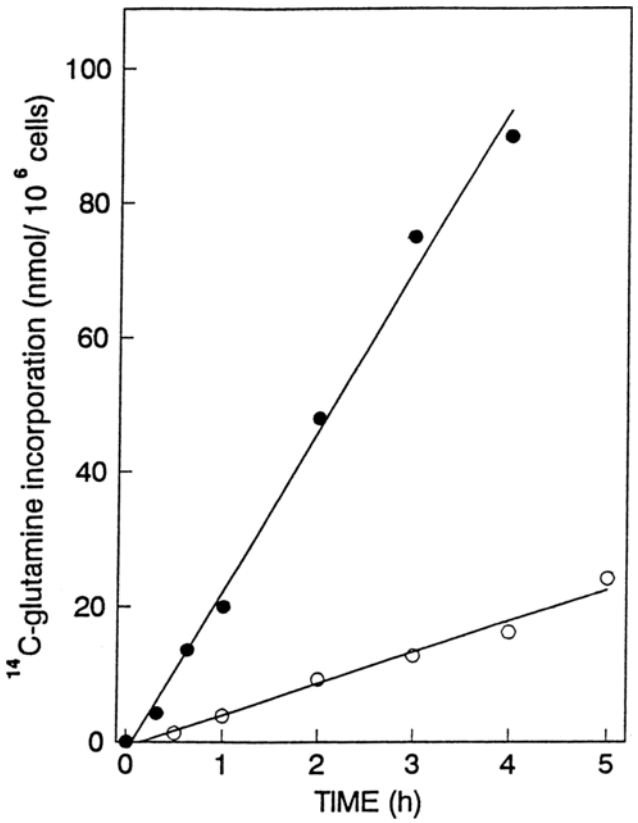

Fig. 7. The incorporation of rate of L-[U-^{14}C]-glutamine into protein and $^{14}CO_2$. The rate of $^{14}CO_2$ release (●) was measured from PQXB1/2 hybridomas (5×10^6/mL) incubated in normal growth medium (1 mL) containing 0.1 μCi of L-[U-^{14}C]-glutamine as described in Materials and Methods. The incorporation of L-[U-^{14}C]-glutamine into a TCA-precipitable fraction (○) was measured by radioactive counting of the precipitate formed by the addition of 50 μL of cold 10% TCA to 50 μL of a cell suspension as described in Materials and Methods. The cells were taken from midexponential phase of batch culture. Each time-point represents the mean of three independent determinations. The standard errors were < 10% of the means in all cases.

The changes in measured enzyme activities over the time period were considerable and reflect the changing composition of the culture. For example, the activity of LDH increased ×4, whereas the activity of PAG decreased ×23. Of the glycolytic enzymes, it is notable that the activities of HK and PFK were relatively low, whereas PK and LDH activities were considerably higher and increased at the end of the culture period. Of the enzymes involved in glutamine metabolism, PAG and GDH activities were particularly low. It is also noticeable that GS activity increased during the stationary phase, which coincided with the time that glutamine was

Table 1
The Flux Through Various Pathways
of Glucose and Glutamine Metabolism[a]

	nmol/min/10^6 cells
Glucose	
Membrane transport	16.0
Pentose phosphate pathway	0.13
TCA cycle	0.008
Glycolysis	3.75
Glutamine	
Membrane transport	1.46
Oxidation to CO_2	0.38
Protein incorporation	0.067

[a]The rates of metabolic flux of glucose and glutamine through various alternative pathways in PQXB1/2 hybridomas were determined from the data shown in Figs. 4–7. The glutamine data were previously reported (*36*).

Table 2
Changes in Specific Enzyme Activities
(nmol/min/10^6 cells) during Growth of PQXB1/2 hybridomas[a]

Enzyme	Day 2	Day 4	Day 7
HK	7.29	4.39	0.82
PFK	60.08	60.45	26.37
PK	192.27	272.74	458.83
LDH	157.08	247.26	630.53
CS	48.45	55.12	16.66
OGDH	12.75	5.85	4.59
PAG	8.87	7.25	0.39
AT	74.97	86.32	42.84
GDH	1.17	0.96	0.99
GS	3.42	1.48	5.20

[a]Specific enzyme activities (nmol/min/10^6 cells) were determined by spectrophotometric assays for 10 enzymes involved in glucose and glutamine metabolism. The enzyme activities were determined in cell extracts from cultures of PQXB1/2 hybridomas taken at three specific time-points in a batch culture as described in Materials and Methods. Each value is based on a mean of three determinations. The standard error in each case is 10–15% of the mean value. Abbreviations: HK = hexokinase; PFK = phosphofructokinase; PK = pyruvate kinase; LDH = lactate dehydrogenase; CS = citrate synthase; OGDH = 2-oxoglutarate dehydrogenase; PAG = phosphate-activated glutaminase; AT = aspartate transaminase; GDH = glutamate dehydrogenase; GS = glutamine synthetase.

Table 3
Potential ATP Production from Glutamine and Glucose in PQXB1/2 Hybridomas Grown in Batch Culture[a]

	ATP from glutamine, nmol/min/10^6 cells			ATP from glucose, nmol/min/10^6 cells		
Day in culture	Gln to Asp, Ala, or Lac	Gln to CO_2	Total	Glucose to lactate	Glucose to CO_2	Total
1	7.53	6.76	14.29	2.96	0.11	3.07
2	15.01	13.47	28.48	7.10	0.27	7.37
3	5.26	4.72	9.98	8.01	0.30	8.31
4	2.71	2.43	5.14	5.07	0.19	5.26
5	3.11	2.79	5.90	0.17	0.01	0.18
6	0.63	0.57	1.20	0.25	0.01	0.26

[a]The maximum potential ATP production was calculated from the utilization of glutamine and glucose over the period of culture of PQXB1/2 hybridomas. The calculations were based on the data of specific consumption rates, the metabolic flux through alternative pathways, and end product analysis as described in Materials and Methods.

exhausted from the medium. No activity was detected for pyruvate dehydrogenase (PDH), which is required to direct pyruvate into the TCA cycle. PDH activity was below the level detectable by a spectrophotometric assay.

Potential Production of ATP

As shown in Fig. 3, the specific rates of substrate utilization change during the culture. This is a reflection of the changing concentration of glucose and glutamine during cell growth. Table 3 indicates the maximum potential ATP production from glucose and glutamine at various timepoints over the period of culture. The distribution of metabolites through the catabolic pathways was calculated with reference to the flux analysis performed with specifically labeled substrates.

The relative importance of glucose and glutamine in providing the potential production of intracellular ATP varies considerably through the culture and is a reflection of the varying utilization rates of the two substrates. At the early and late stages of the culture, glutamine is capable of providing a considerable proportion (> 80%) of ATP, whereas during the exponential growth phase at days 3 and 4, when the glucose consumption is maximum, the potential provision of ATP is equally balanced between the two substrates.

DISCUSSION

The motivation for analyzing the energy metabolism of a hybridoma is for an improved understanding of nutrient requirements and utilization

during growth and antibody production in batch culture. Although such cultures are used for commercial production of antibodies, there have been few studies of the energy metabolism of these cells. The cellular energy required for product formation is a substantial fraction of the total energy capacity of the cell. Thus, if ATP synthesis is rate-limiting, high growth rates may retard product formation (*3*).

Glucose and glutamine are the major energy substrates for most cells grown in culture, and the metabolism of these two substrates is interactive. Glucose (5–10 m*M*) can decrease the utilization of glutamine by cells in culture (*6,44,45*). On the other hand, glutamine can stimulate glucose utilization via the glycolytic and pentose phosphate pathways, but inhibit its oxidation in the TCA cycle (*43,44,46*). When both glucose and glutamine are present in lymphocyte culture, the utilization rates of both substrates increase significantly (*13*).

Changes During Culture

The murine hybridoma, PQXB1/2 used in the present study undergoes a maximum growth rate in culture for three days during which the intracellular protein content increases ×3. This is consistent with a high proportion of the cell population in the mitotic (M) and synthetic (S) phases of the growth cycle.

The pattern of nutrient utilization and byproduct formation for the PQXB1/2 cells is typical of mammalian cells in batch culture (*4*). Glutamine is completely depleted and glucose partially depleted when the cells enter a stationary phase. Although further cell growth may be limited at this stage by the complete utilization of glutamine, there may also be some growth inhibitory effect owing to the accumulation of ammonia. The ammonia concentration resulting in 50% growth inhibition has been previously determined for the PQXB1/2 cells as 5.1 m*M* (*47*). Although the maximum ammonia concentration was only 1.5 m*M* in batch cultures of PQXB1/2 cells, this could still be growth inhibitory.

The specific rates of substrate utilization are high during the growth phase of the PQXB1/2 cells when both glucose and glutamine are available in the medium. The maximum rates of substrate utilization are considerably higher than previous reports of lymphocytes (*13*), thymocytes (*38*), or splenocytes (*11*) isolated from primary sources. This is probably a reflection of the high growth rate and antibody production of the PQXB1/2 cells in batch culture. After 4–5 d of culture, the utilization rates decreased to zero. This coincides with the time of complete utilization of glutamine, but only the partial utilization of glucose (50% of the original concentration).

The maximum catalytic activities of some of the enzymes of glucose and glutamine metabolism were determined at three time-points in the PQXB1/2 cultures. The activities were within the same order of magnitude as those previously measured in rat lymphocytes using similar assays (*16*).

The changes in enzyme activities during batch culture occur in response to intracellular regulators or to the effect of the changing availability of substrates in the culture medium. Many, although not all, of the enzyme activities were highest during the exponential growth phase. This compares with a previous study showing increased enzyme activities (×3 to ×30) associated with the activation of resting thymocytes to proliferation (*48*).

Glucose Metabolism

The pattern of glucose metabolism was determined at midexponential growth phase by use of specifically radiolabeled substrates. These show a high rate of flux through glycolysis (96% of glucose utilized), but only a limited conversion into the TCA cycle (0.21%). This is consistent with the high rate of anaerobic glycolysis previously reported in CHO cells (*49*), rat thymocytes (*38*), and rat splenocytes (*11*).

The determined lactate/glucose coefficient at 2.05 mol/mol was slightly higher than the theoretical maximum for lactate formation from glucose and certainly indicates that a high proportion of glycolytic metabolites was converted to lactate. Substrates other than glucose may also be converted to lactate (*50*). For example, glutamine, other amino acids, or TCA cycle intermediates could be transformed to glycolytic intermediates via the malate and PEP-carboxykinase enzymes. Other known end products of glycolysis include alanine, which is secreted at a relatively high rate (1.2 nmol/min/10^6 cells) during the growth of PQXB1/2 hybridomas, particularly in response to added ammonia (*51*). Alanine production from pyruvate was suggested as a mechanism of intracellular detoxification of ammonia.

The pentose phosphate pathway flux was measured by the difference in release of $^{14}CO_2$ from C-1- and C-6-labeled glucose substrates. The results show that 3.3% of the metabolized glucose follows this route. This pathway has been shown to be essential for dividing cells for the synthesis of ribose phosphate, which is a precursor of nucleic acids (*44*). In fact, it has been suggested that this is the only essential function of glucose metabolism (*52*). In some cultured cells, it has been shown that glucose is not essential if a pentose source is provided in the medium (*8*).

The rate-limiting steps of metabolic pathways can be identified by analysis of maximum enzyme or transport activities (*15*). The maximum activity of a rate-limiting enzyme can indicate the maximum flux possible for a pathway.

The measured rate of glucose transport into the PQXB1/2 cells was significantly higher than the utilization rates in culture or the measured rates of metabolic flux for glucose. This indicates that glucose transport is unlikely to be limiting to glucose metabolism in these cells. Similar glucose transport rates were reported for chick fibroblasts, which showed considerably enhanced rates following glucose starvation (*35,53*).

The specific hexokinase (HK) activity at 4.39 nmol/min/10^6 cells is close to the glucose consumption rate at the midexponential phase. The HK activity decreases to 11% of the original value at the end of the growth period and is coincident with the stationary phase. It is proposed that the activity of HK may determine the overall rate of glucose metabolism, and its reduction to a low activity at the end of the growth period may be responsible for the low utilization rate of glucose, even though the substrate is still available in the medium at 50% of its original concentration.

It was previously reported that the mitogenic activation of rat thymocytes results in the stimulation of glycolysis by the coordinated activation of glucose transport and phosphofructokinase (PKF) activities (*38*). This suggested that PFK could be a regulatory enzyme. However, the activities of PFK determined in the PQXB1/2 cells show that they are well above the measured flux value for glycolysis, even though the activity is reduced by over 50% in the later part of the culture. Glutamine has been shown to stimulate the activity of PFK (*13*). It may be that in the PQXB1/2 cultures reported here, the depletion of glutamine caused the decreased activity of PFK in the later stage of culture. A similar mechanism may also be proposed for the decrease in HK activity, which appears to be regulatory for glucose utilization.

Pyruvate kinase (PK) and lactate dehydrogenase (LDH) activities are high compared to those of HK and PFK, and are unlikely to regulate the flux through glycolysis in the PQXB1/2 cultures. PK and LDH activities increased by factors of x2.4 and x4, respectively, during the culture (Table 2). This was in contrast to the HK and PFK activities, which decreased over this period. The PK/HK activity ratios have often been quoted as an indication of the relative importance of the two enzymes in glycolytic regulation. Previously determined PK/HK values vary from 13 for lymphocytes (*16*) and 30 for glioma cells (*54*) to 258 for skeletal muscle (*55*). The activity ratios determined for PQXB1/2 cells varied from 26 on day 2 to 560 on day 7.

The change of intracellular activity of LDH for the PQXB1/2 cells contrasts with findings for human tumor kidney cells (*56*) in which the LDH activity was reported to be stable over time. The extracellularly released LDH has been suggested as an indicator of cell viability, but this method would be invalid for cells such as PQXB1/2, which show large changes in specific LDH activity during culture.

An activity for pyruvate dehydrogenase (PDH) was not detected in the PQXB1/2 cells by using a spectrophorometric assay. This suggests a low activity of PDH, which would provide a barrier for glycolytic intermediates entering the TCA cycle. Enhancement of the activity of this enzyme could well be a means of promoting the more efficient aerobic metabolism of glucose.

The activities of two TCA cycle enzymes, citrate synthase (CS) and oxoglutarate dehydrogenase (OGDH), were determined and found to be considerably higher than rates that would be expected to be limiting to

metabolic flux. The activity of OGDH correlated with the TCA cycle flux rate in rat muscle (*15*). However, in the PQXB1/2 cells, OGDH activity was several orders of magnitude greater than the measured rate of flux of glucose carbon through the TCA cycle and 10x greater than the total rate of glutamine metabolism. This suggests that these two enzymes do not regulate the TCA cycle flux.

Glutamine Metabolism

In these studies, the importance of glutamine is suggested by the observation that glutamine, but not glucose is depleted from the medium when the cells enter a stationary phase. Glutamine can be used as an energy substrate following deamination of the molecule to the TCA cycle intermediate, 2-oxoglutarate. This can in turn be completely oxidized to CO_2 by the TCA cycle or partially oxidized to aspartate, alanine, or lactate. In fact, eight independent metabolic pathways have been described for glutaminolysis (*41*). Measurement of glutamine oxidation in the PQXB1/2 cells showed that 36% of glutamine carbon utilized by the cells at the midexponential phase was converted to CO_2 (*36*).

The measured rate of glutamine transport approximates to the specific rate of glutamine utilization by the cells on the first day of culture when the glutamine concentration is 2 m*M*. This suggests that glutamine transport may be the limiting factor in glutamine metabolism in these cells. The higher rate of glutamine utilization at a later stage of culture may be explained by induction of the glutamine transport mechanism, which may occur at lower glutamine concentrations (*57*).

The first enzyme required for glutaminolysis is phosphate-activated glutaminase (PAG), which has been considered as a rate-limiting step in glutamine metabolism in some cells. The activity of the enzyme is regulated by the intracellular phosphate concentration, which may be reduced following glycolytic ATP synthesis (*58*). This may explain the reduction in activity of this enzyme by a factor of nearly x23 in the later phase of the PQXB1/2 culture, a phenomenon also reported in human diploid fibroblast cultures (*59*). The initial activity measured for PAG in PQXB1/2 cells indicates a value well above the rate of glutamine utilization and suggests that at the early stage of the culture, this enzyme is unlikely to be rate-limiting. This concurs with previously reported data for lymphocytes (*16*).

Removal of the amino group of glutamate is the second step of glutaminolysis. This occurs by oxidative deamination with glutamate dehydrogenase (GDH) or by transamination with an aminotransferase. Of the various aminotransferase enzymes, alanine transferase and aspartate transaminase are the most likely to be involved in the transamination of glutamate. Both alanine and aspartate have been shown to be major by-products of cultured hybridomas or lymphocytes (*10,51*). The maximal activity of aspartate transaminase was measured in the PQXB1/2 hybrid-

oma and is considerably higher than GDH throughout the culture period. This would argue for the transamination reaction as the major route for glutamate conversion. The measured coefficient of 0.6 mol/mol for ammonia/glutamine also supports this metabolic route. Kinetic studies in other mammalian cells suggest that transamination is the predominant pathway for conversion of glutamate carbon to 2-oxoglutarate (*16,60*).

The regulation of the enzyme glutamine synthetase has been well studied (*57,61*) and has shown that its activity depends on the glutamine concentration in the medium. The increase in GS activity in the stationary phase of the cultures reported here is compatible with previous data that increased activity occurs when the concentration of glutamine is decreased in the medium.

Potential Production of ATP

The relative importance of glucose and glutamine in providing intracellular energy as ATP has been assessed previously by various studies (*10,11,46*). The stimulation of lymphocytes by a mitogen causes a significant increase in the rate of glutamine metabolism. In such stimulated cells, the relative contribution of glutamine metabolism as a provider of intracellular ATP is increased in comparison to resting cells (*11*). This enhancement together with the high basal level of glutamine metabolism has been proposed as a mechanism for the ability of these cells to proliferate rapidly in response to immunogenic stimulation in vivo (*62*). In the cultures described here, the PQXB1/2 hybridomas have a high growth rate and continuously synthesize antibody. In that respect, they may be compared with the mitogenically stimulated lymphocytes analyzed in previous work.

The relative contribution of glucose and glutamine metabolism in the provision of intracellular energy was estimated for PQXB1/2 cells from the data on metabolic flux. These calculations indicate that in the early stage of culture, glutamine is the major contributor of ATP. However, there is a period in the mid- to late-exponential phase when the contributions from glucose and glutamine metabolism are finely balanced. For example, at day 3, it was estimated that glutamine provided 55% of cellular energy and glucose 45%. Similar estimations of ATP production were made by Wu et al. (*10,11*) for glucose and glutamine metabolism in rat splenocytes and mesenteric lymphocytes. They also concluded that each substrate could potentially contribute equal amounts of intracellular ATP, although apparently contradictory results were obtained by O'Rourke and Rider (*46*), who concluded that glutamine metabolism makes only a minor contribution to ATP generation in rat splenocytes. Various studies with other cells have concluded that the relative contribution of glutamine to cellular energy is between 30 and 98%, depending on the cell line and substrate concentrations (*6,49,50,63*).

Metabolic Management

Various suggestions have been made for the metabolic management of cells in order to enhance productivity in culture. An enhancement of oxidation of glycolytic metabolites would promote the more efficient utilization of glucose and reduce lactate production. Lactate is produced as a major product in the cultures reported here because of the high LDH/PDH activity ratio, which would promote the conversion of pyruvate to lactate. The activation of PDH has been suggested by the inclusion of dichloroacetate in the culture medium (*64*). This has been shown to enhance specific antibody production. A second previously suggested strategy is the addition of inhibitors of GDH in order to enhance the activity of aminotransferases with respect to GDH (*3*). This would minimize the ammonia produced per mole of ATP. The ratio of AT/GDH activities is relatively high in the PQXB1/2 cells, but it would be of interest nevertheless to determine the effect of further increasing this activity ratio.

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