

## Uptake of glutamate, not glutamine synthetase, regulates adaptation of mammalian cells to glutamine-free medium

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### SUMMARY

Two cell lines (McCoy and MDCK) were studied in an attempt to understand the metabolic changes associated with adaptation to glutamine-free medium (GMEM + gmate). McCoy cells assumed normal growth rates after 2-3 passages in this medium whereas MDCK cells showed no growth in GMEM + gmate.

The glutamine synthetase (GS) activity of both cell lines was elevated (up to  $\times 9$ ) as glutamine was depleted from normal media (GMEM + gmine). The high activity of GS was maintained during McCoy cell growth in GMEM + gmate. However, there was no apparent significant difference between the two cell lines in the pattern of changes of GS activity in response to glutamine.

The cellular uptake rates of glutamine and glutamate from the medium differed significantly between the two cell lines. During the adaptation of McCoy cells to GMEM + gmate, the rate of glutamate uptake doubled to a value of 0.54 nmol/min per mg cell protein whereas the maximum value for MDCK cells was considerably lower (0.04 nmol/min per mg cell protein). We propose that the difference in intrinsic ability for glutamate transport accounts for the difference in growth response between the two cell lines in the glutamine-free medium.

Key words: cell culture, adaptation, glutamine, glutamic acid, glutamine synthetase

### INTRODUCTION

The growth of mammalian cells to high densities is essential for the development of culture processes required in the large-scale production of biological compounds. Achievement of high cell yields in such cultures is often limited by an accumulation of ammonia (Butler et al., 1983; Butler and Spier, 1984), although cells can vary in their tolerance to this metabolic by-product (Hassell et al., 1991).

Ammonia is generated in culture from the metabolic utilisation of glutamine (Reitzer et al., 1979) and by its chemical decomposition in the medium (Tritsch and Moore, 1962). Cell culture media in general usually contain relatively high concentrations of glutamine (2-4 mM). This derives from the original media formulations which were based upon observed cellular requirements (Eagle, 1955, 1959). Glutamine is required as a substrate and precursor for protein synthesis, nucleotide synthesis and energy metabolism (Zielke et al., 1984; Wice et al., 1981).

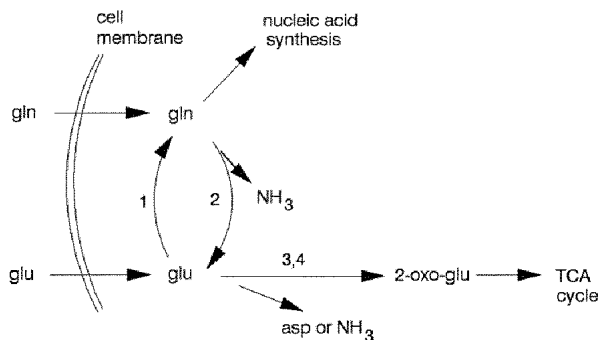
However, there are several disadvantages connected with the use of glutamine. Because of its thermal instability, glutamine is generally added to media as a separate, filter-sterilised supplement immediately prior to use. Storage of glutamine-based media, even at 4°C, can result in significant decomposition and undesirable ammonia accumulation even after relatively short periods (Tritsch and Moore,

1962). Furthermore, as indicated earlier, maximum cell yields in culture can be limited by excessive glutamine metabolism resulting in toxic levels of ammonia. Thus, the development of a glutamine-free medium which could support cell growth would be attractive.

One approach to the formulation of a glutamine-free medium is to utilise a glutamine analogue which is less ammoniagenic. There are numerous reports of the ability of high concentrations (20 mM) of glutamic acid to replace glutamine (2-4 mM) in supporting the growth of some cell lines (Eagle et al., 1956; Griffiths and Pirt, 1967; Griffiths, 1973). However, the ability of cells to adapt to glutamine-free medium is variable.

In a recent report (Hassell and Butler, 1990) it was shown that some cells assumed normal growth rates in glutamine-free medium within 2-3 passages, whereas other cell lines failed to adapt or take considerably longer periods. Following adaptation, the glutamine-free cultures produced lower levels of ammonia and consequently higher cell yields.

Fig. 1 illustrates the key enzymatic reactions associated with glutamine/glutamate metabolism and the associated production of ammonia. If the cells utilise glutamate in the absence of glutamine, then intracellular levels of glutamine must be synthesised using the enzyme, glutamine synthetase. The mechanism of adaptation to glutamine-free



**Fig. 1.** The key steps of glutamine and glutamate metabolism. (1) Glutamine synthetase (GS). (2) Phosphate-activated glutaminase (PAG). (3) Glutamate dehydrogenase (GDH). (4) Glutamate: oxaloacetate transaminase (GOT).

medium has been thought to be closely associated with the observed increase in intracellular activity of glutamine synthetase (Griffiths, 1973), which enhances the capacity for glutamine synthesis. The repression and de-repression of this enzyme by glutamine and glutamate was described in some early papers (DeMars, 1958; Paul and Fottrell, 1963). However, no adequate explanation has been offered for the variation in ability to adapt between cell lines.

The purpose of the work described in this paper was to investigate some of the metabolic changes associated with the adaptation of cells to glutamine-free media and to establish the basis for the differences between cell lines in the ability for adaptation. The two cell lines (McCoy and MDCK) were chosen for study and comparison in this investigation because of their observed differences in response to glutamine-free media.

## MATERIALS AND METHODS

### Cells

The cell lines used were McCoy and MDCK (Madin Darby canine kidney). McCoy cells originated from mouse LS fibroblasts, and were kindly donated by Mr A. Bailey of Booth Hall Children's Hospital, Manchester. Madin-Darby canine kidney (MDCK) cells are derived from renal epithelial cells and were obtained from Flow Laboratories, Scotland, UK. Cells were shown to be free of mycoplasma contamination by routine testing.

### Culture medium

The growth medium used for both cell lines was the Glasgow modification of Eagle's medium (GMEM; Stoker and MacPherson, 1964), supplemented with glutamine at 2 mM (GMEM + gmine) or glutamic acid at 2 mM (GMEM + gmate). The glucose concentration was 25 mM in all cases.

The medium was also supplemented with 10% (v/v) heat inactivated newborn calf serum. Medium and serum was 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.

### Culture

Cells were inoculated into 10 ml culture medium contained in 25

cm<sup>2</sup> T-flasks or 50 ml culture medium in 150 cm<sup>2</sup> T-flasks (Corning). The flasks were incubated at 37°C in a 10% CO<sub>2</sub> atmosphere.

### Cell harvest

Cells were harvested from T-flasks by the addition of 0.25% trypsin in PBS for 5 min (McCoy cells) or by trypsin-EDTA (0.05% trypsin/0.02% EDTA) for 15 min (MDCK cells). The detached cells were centrifuged at 1,000 *g* for 5 min before resuspension in fresh medium.

### Cell enumeration

Cell concentrations were determined by microscopic counting using a Neubauer haemocytometer.

### Radioactive amino acid uptake

Cells were inoculated into 24-well plates at  $1 \times 10^4$  cells/ml. After 96 h of culture, rates of glutamine or glutamic acid uptake were determined by a method based on that described by Forster and Lloyd (1985). Culture medium was removed by aspiration and the attached cells were washed ( $\times 3$ ) with cold PBS. The cells were then equilibrated at 37°C in 200  $\mu$ l of GMEM + 10% serum. Measurement of transport was initiated by the addition of L-[U-<sup>14</sup>C]glutamine (2 mM, specific activity 0.25 Ci/mol) or L-[U-<sup>14</sup>C]glutamate (2 mM, specific activity 1.0 Ci/mol).

Cultures were incubated at 37°C in a 10% CO<sub>2</sub> atmosphere for various time intervals up to 10 min. Transport was terminated by aspiration of the radiolabelled incubation medium and the cells were rinsed three times in ice-cold PBS. The cells were dissolved in 150  $\mu$ l of 0.5 M NaOH and a sample (100  $\mu$ l) was measured for radioactivity using Ecoscint A scintillation fluid and an LKB Rackbeta II liquid scintillation counter.

The radioactivity of the intracellular content of each cell sample was calculated following determination of the extracellular and intracellular space. These relative volumes were determined in separate incubations with <sup>3</sup>H<sub>2</sub>O and [<sup>3</sup>H]inulin. <sup>3</sup>H<sub>2</sub>O readily enters the cells and allows an estimation of total space. [<sup>3</sup>H]inulin does not enter the cells and allows an estimation of the extracellular space. In our experiments the estimated extracellular space was between 62% and 78% of the total cell extract.

### Cell extracts for protein and enzyme analysis

A cell suspension was washed twice in cold PBS and resuspended in extraction buffer (0.15 M KCl, 2 mM dithiothreitol, 1 mM EDTA, pH 7.2). The cells were disrupted either by: (a) sonication for  $4 \times 15$  s bursts at an amplitude of 7-8  $\mu$ m peak-to-peak (Sonicator No. C006/66, F.T.Z. Seriiën-Prof., Nr.) with cooling intervals of 1 min or (b) 3 freeze-thaw cycles at temperature of liquid nitrogen and 37°C. After centrifugation at 6,500 *g* for 5 min the supernatant was assayed for protein content or for enzyme activity.

### Glutamine synthetase assay

The GS activity of cell extracts was measured by one of two assays.

#### Colorimetric assay

This is based upon the formation of  $\alpha$ -glutamylhydroxamate from glutamine and hydroxylamine (Kim and Rhee, 1987). A coloured complex is then formed with ferric chloride, which can be measured colorimetrically. This is a measure of glutamyltransferase which has been shown to reflect glutamine synthetase activity.

Cell extracts (100  $\mu$ l) were incubated at 37°C with 0.1 M imidazole-HCl, 50 mM L-glutamine, 0.4 mM MnCl<sub>2</sub>, 62.5 mM hydroxylamine and 10 mM sodium arsenate in a final volume of

500  $\mu$ l (pH 7.2). The reaction was terminated by the addition of 1 ml  $\text{FeCl}_3$  reagent (0.37 M  $\text{FeCl}_3$ , 0.67 M HCl, 0.2 M TCA). Precipitated protein was removed by centrifugation at 10,000 g for 5 min and the absorbance of the supernatant was measured at 535 nm.

### Radioactive assay

GS activity was also assayed by the conversion of L-[U- $^{14}\text{C}$ ]glutamic acid to L-[U- $^{14}\text{C}$ ]glutamine (Smith et al., 1984). A sample of cell extract containing between 25  $\mu$ g and 100  $\mu$ g protein was incubated with 2.5 mM L-[U- $^{14}\text{C}$ ]glutamic acid (0.68 Ci/mol), 7.5 mM ATP, 30 mM  $\text{MgCl}_2$ , 25 mM  $\text{NH}_4\text{Cl}$  and 50 mM imidazole-HCl buffer (pH 7.5) in a final volume of 250  $\mu$ l. After incubation for 60 min at 37°C the reaction was terminated by addition of 1 ml 20 mM imidazole-HCl, pH 7.5, at 4°C. A sample containing 1 ml of the resulting suspension was applied to a 1 ml bed volume column of Dowex 1-X8 anion-exchange resin (200-400 mesh, 8% crosslinked, chloride form) that had previously been equilibrated with 20 mM imidazole-HCl, pH 7.5. Elution with the same buffer enabled separation of the glutamine fraction from that of glutamate, which was then quantified by scintillation counting.

### Glutamine determination

L-glutamine was determined as ammonia after conversion with glutaminase (*E. coli*). Each media sample (125  $\mu$ l) was incubated with 100  $\mu$ l sodium acetate buffer (0.1 M, pH 4.9) and 25  $\mu$ l glutaminase (5 i.u./ml in 0.005 M sodium acetate buffer, pH 6.0) for 15 min at 37°C. After deproteinisation with 20% PCA, ammonia concentrations were determined colorimetrically (based on the method of Gutmann and Bergmeyer, 1974).

### Glutamate determination

L-glutamate concentrations were determined by oxidative deamination to 2-oxoglutarate in the presence of NAD and glutamate dehydrogenase (Beutler and Michal, 1974). In the presence of diaphorase, the resulting NADH reduces iodionitrotriazolium chloride (INT) to a formazan, whose absorbance can be measured at 492 nm.

After deproteinisation with 20% PCA, each media sample (500  $\mu$ l in a final volume of 3.5 ml) was mixed with triethanolamine (57 mM), potassium phosphate (14 mM, pH 8.6), NAD (0.38 mM), INT (0.068 mM), diaphorase (0.14 i.u./ml) and GDH (14 i.u./ml). The change in absorbance at 492 nm was measured against a reagent blank.

### Protein determination

Protein concentrations of cell extracts were determined colorimetrically by the Bradford reagent (Bradford, 1976).

## RESULTS

### Adaptation of cells to glutamine-free medium

McCoy cells were adapted to glutamine-free medium by harvesting the cells at confluence and substituting GMEM containing 2 mM glutamine (GMEM + gmine) with GMEM containing 2 mM glutamate (GMEM + gmate). The growth rate in GMEM + gmate assumed a normal doubling time of 20 h after 2-3 passages.

All attempts to adapt MDCK cells to GMEM + gmate failed. Several protocols were attempted. A substitution of GMEM + gmine with GMEM + gmate resulted in no cell growth. An alternative protocol in which cells were inoculated into GMEM containing a high concentration (20 mM)

of glutamate also failed. A gradual reduction of glutamine concentration in conjunction with an increase of glutamate enabled MDCK cells to grow in GMEM containing 0.5 mM glutamine and 1.5 mM glutamate (GMEM + gmine/gmate). However, even after 10 passages in GMEM + gmine/gmate the growth rate corresponded to a doubling time of 31 h, which was considerably greater than the normal 18 h doubling time in GMEM + gmine.

Fig. 2 shows the effect of substituting GMEM + gmine for GMEM + gmate after 2 days of growth of McCoy cells (Fig. 2A) and MDCK cells (Fig. 2B) in T-flasks. The McCoy cells continued growth in GMEM + gmate although at a reduced growth rate, but the MDCK cells showed no increase in cell concentration after the medium substitution.

The difference in the ability of these two cell lines to adapt to glutamine-free medium formed the basis of this investigation into the mechanism of adaptation.

### Glutamine synthetase activity during cell growth

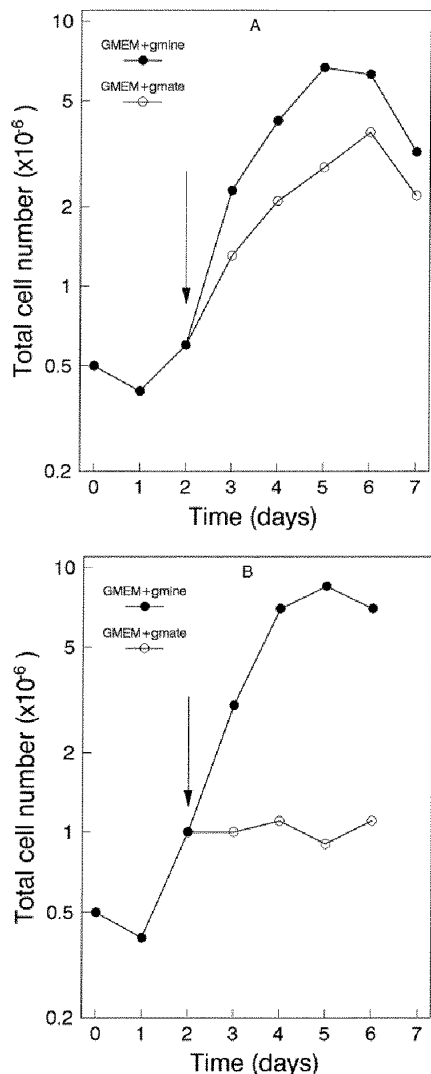
Changes in GS activity have been associated with the concentration of glutamine in the medium and these were investigated. The intracellular glutamine synthetase activity of McCoy (Fig. 3A) and MDCK cells (Fig. 3B) was determined by colorimetric assay over a 7-day growth period in GMEM + gmine. The glutamine was completely utilised by either cell line after 6 days, a time period which corresponds to the limit of cell growth. There was a significant increase in GS activity ( $\times 9$ ) for the McCoy cells during this period. A similar pattern was shown for the change in intracellular GS activity of MDCK cells. The initial glutamine synthetase activity of MDCK cells was slightly higher than McCoy cells and increased by 5.5. The pattern of glutamine synthetase activity increase in the absence of glutamine appeared to be common to both cell lines, with no significant difference in the final elevated levels: 9 nmol/min per mg protein for McCoy cells and 8 nmol/min per mg cell protein for MDCK cells.

A similar analysis was performed for McCoy cells grown in GMEM + gmate (Fig. 4). The GS activity of these adapted cells (6 nmol/min per mg cell protein) was significantly higher than cells grown in fresh GMEM + gmine, although slightly lower than the level found in GMEM + gmine when the glutamine was completely depleted. No significant changes in intracellular GS activities occurred during growth in GMEM + gmate, despite a rate of glutamate consumption similar to that of glutamine in GMEM + gmine.

### Glutamine synthetase activity following rapid changes in glutamine concentration

To determine the kinetics of response of glutamine synthetase to changes in concentration of glutamine or glutamate, the activity of the enzyme was monitored over a short time span following the addition of either amino acid.

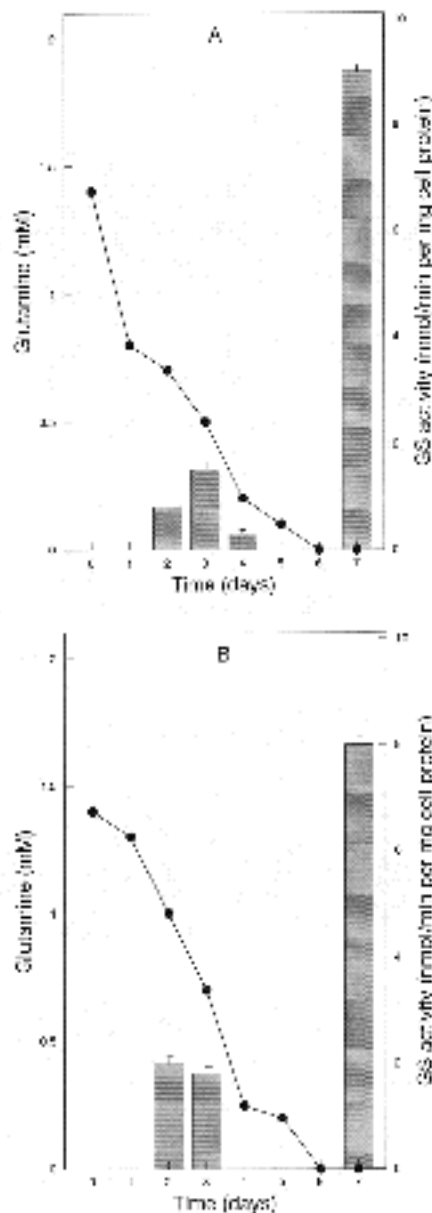
In this experiment, McCoy or MDCK cells were inoculated into GMEM + gmine and grown for 7 days, after which time glutamine was depleted from the media. The medium was then exchanged for fresh GMEM + gmine. In parallel cultures 2 mM glutamine was added to the medium after 6 days. At day 7 the medium in these cultures was



**Fig. 2.** Growth of McCoy and MDCK cells in GMEM + gmine and GMEM + gmate. McCoy cells (A) or MDCK cells (B) were inoculated ( $5 \times 10^4$  cells/ml) into replicate cultures of GMEM + gmine (10 ml) contained in T-flasks ( $25 \text{ cm}^2$ ). After 48 h (arrow), the medium in the flasks was replaced by fresh GMEM + gmine (●) or by GMEM + gmate (○). Each point represents the mean of three values from independent cultures. In all cases the standard error was less than 10% of the mean.

replaced by GMEM + gmate. The glutamine synthetase activity was measured from cell extracts of all cultures over a 45 h period following medium exchange at day 7.

Fig. 5 shows that there was a difference of 2- to 3-fold in the GS activity depending upon the addition of glutamine 24 h prior to monitoring the enzyme levels. A lower GS activity was associated with the addition of glutamine. At time zero the cells with an elevated GS activity were treated with the glutamine-based medium (GMEM + gmine) and this caused a rapid decrease (2- to 3.5-fold) in the enzyme activity over 5 h. Cells with the lowered GS activity were treated with the glutamate-based medium (GMEM + gmate) and this caused a 3-fold increase in enzyme activity in the McCoy cells and a 1.5-fold increase in the MDCK cells, both reaching maximal activities after

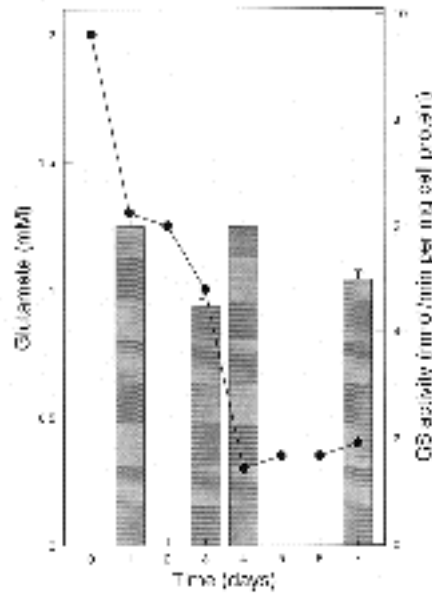


**Fig. 3.** Glutamine synthetase activities of McCoy and MDCK cells during growth in the presence of glutamine. McCoy cells (A) or MDCK cells (B) were inoculated ( $4 \times 10^4$  cells/ml) into T-flasks ( $150 \text{ cm}^2$ ) and grown in GMEM + gmine (50 ml). Glutamine concentrations (●) in the culture medium were determined and intracellular glutamine synthetase activity (columns) was assayed during the culture period. Each point represents the mean of three independent values.

20 h. Although there were slight differences in the measured activity levels between the two cell lines, there were no apparent differences in the pattern of changes.

### Changes in the kinetic properties of glutamine synthetase during cell growth

The kinetics of glutamine synthetase activity as a function of glutamate concentration were studied from cell extracts taken during the growth of MDCK cells in GMEM + gmine and McCoy cells in GMEM + gmine and GMEM + gmate.



**Fig. 4.** Glutamine synthetase activities of McCoy cells during growth in the presence of glutamic acid. McCoy cells were inoculated ( $4 \times 10^4$  cells/ml) into T-flasks ( $150 \text{ cm}^2$ ) and grown in GMEM + gmate (50 ml). Glutamate concentrations ( $\bullet$ ) in the culture medium were determined and intracellular glutamine synthetase activity (columns) was assayed during the culture period. Each point represents the mean of three independent values.

Cells were cultured in T-flasks ( $150 \text{ cm}^2$ ) and harvested at various time intervals throughout the growth period. The GS activity of cell extracts was determined by the radioactive enzyme assay involving conversion of L-[U- $^{14}\text{C}$ ]glutamate to L-[U- $^{14}\text{C}$ ]glutamine.

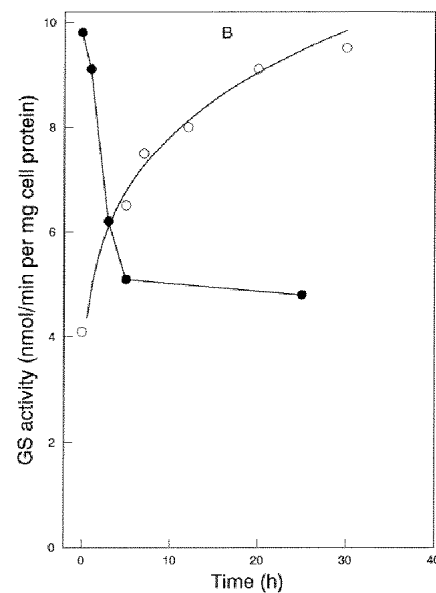
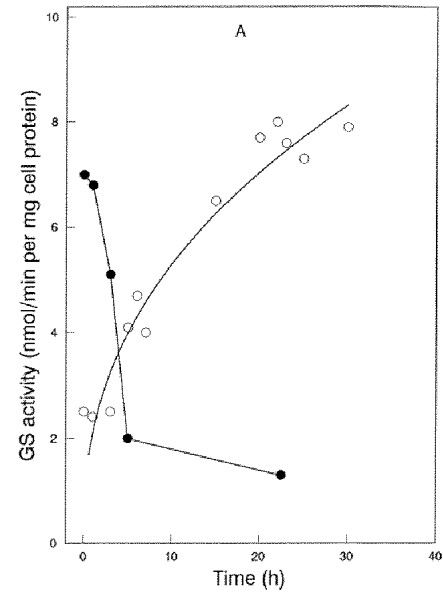
The data obtained from this experiment are expressed as Michaelis-Menten plots from which  $K_m$  and  $V_{max}$  values could be determined for each time point (Tables 1 and 2). This showed that for McCoy and MDCK cells grown in GMEM + gmine, the  $V_{max}$  value of GS increased with the rapid consumption of glutamine from the medium and confirms the previous results by the colorimetric assay. The Michaelis constant ( $K_m$ ) of glutamine synthetase also changed during this period from an initial value of 4.5 mM glutamate to 2.5 mM glutamate, with respect to both these cell lines. In the case of McCoy cells grown in GMEM + gmate, the  $V_{max}$  of GS was relatively high and the  $K_m$  relatively low but there were no apparent significant changes in the enzymatic parameters of GS during cell growth.

#### Uptake of glutamine and glutamate into cells

The rates of uptake of L-[U- $^{14}\text{C}$ ]glutamine and L-[U- $^{14}\text{C}$ ]glutamate were determined for McCoy cells adapted to GMEM + gmine or GMEM + gmate and MDCK cells grown in GMEM + gmine. The protocol for determining uptake is described in the Materials and methods section.

Examples of results from the time course of uptake of radiolabelled glutamine and glutamate are shown in Fig. 6. A summary of the rates of uptake determined from these experiments is shown in Table 3.

The uptake of glutamine was linear for at least 2 min



**Fig. 5.** Rapid changes in intracellular glutamine synthetase activity of (A) McCoy and (B) MDCK cells following addition or removal of glutamine. Addition of glutamine. Cells ( $4 \times 10^4$ /ml) were inoculated into GMEM + gmine (50 ml) in T-flasks ( $150 \text{ cm}^2$ ) for 7 days at which point the glutamine of the medium had been depleted and the intracellular glutamine synthetase activity was elevated (Fig. 4). At time zero the medium was exchanged for fresh GMEM + gmine and changes in the intracellular glutamine synthetase activities were determined over a 24 h period as shown ( $\bullet$ ). Removal of glutamine. Cells were grown in GMEM + gmine in T-flasks ( $150 \text{ cm}^2$ ) for 6 days, after which glutamine (2 mM) was added to each flask. After a further 24 h, the cells were washed twice in PBS and the medium in all flasks exchanged for GMEM + gmate. Changes in the intracellular glutamine synthetase activities were then determined for a period of 30–40 h as shown ( $\circ$ ). Each point represents the mean of three independent experiments and in all cases the standard error was less than 10% of the mean.

**Table 1. Apparent  $K_m$  values (mM) with respect to glutamate determined for glutamine synthetase extracted from McCoy and MDCK cells at different points during culture**

Time (h)	Cell line		
	McCoy (GMEM+gmine)	MDCK (GMEM+gmine)	McCoy (GMEM+gmate)
48	4.93 ± 1.73	4.04 ± 0.72	2.63 ± 0.34
72	4.24 ± 0.87	4.34 ± 0.64	nd
96	nd	nd	3.57 ± 0.54
120	nd	2.22 ± 0.16	nd
144	2.88 ± 0.18	nd	2.71 ± 0.32

Cells were grown in GMEM + gmine or GMEM + gmate (see text), for the indicated time. Values are the mean of two experiments in each case. nd, not determined.

**Table 2. Apparent  $V_{max}$  values (nmol/min per mg cell protein) determined for glutamine synthetase extracted from McCoy and MDCK cells at different points during culture**

Time (h)	Cell line		
	McCoy (GMEM+gmine)	MDCK (GMEM+gmine)	McCoy (GMEM+gmate)
48	0.60 ± 0.12	1.86 ± 0.17	2.75 ± 0.14
72	1.25 ± 0.17	3.49 ± 0.27	nd
96	nd	nd	3.50 ± 0.26
120	nd	4.29 ± 0.11	nd
144	2.52 ± 0.07	nd	2.99 ± 0.14

Cells were grown in GMEM + gmine or GMEM + gmate (see text) for the indicated time. Values are the mean of two experiments in each case. nd, not determined.

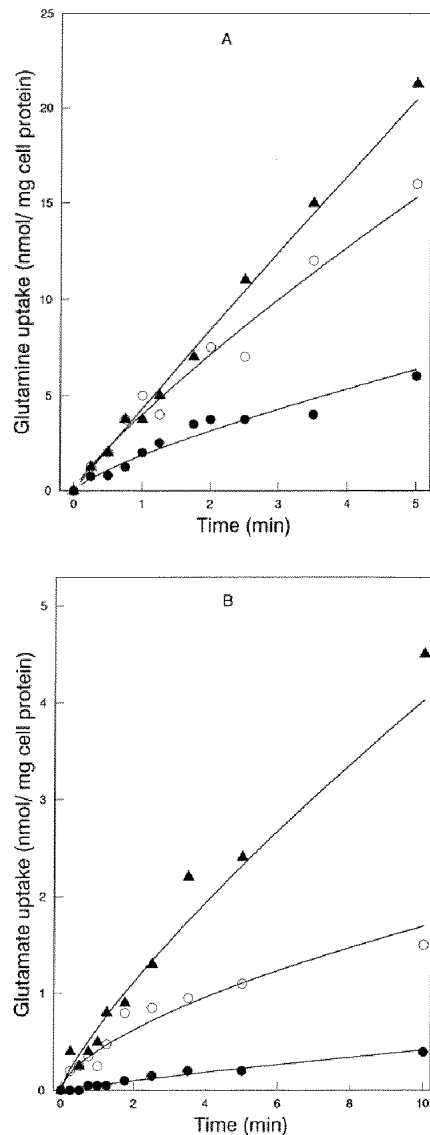
and an initial uptake rate was determined for this time period. These initial rates represent rates of membrane transport. The rate of transport of glutamine by MDCK cells was significantly lower than for McCoy cells in either GMEM + gmine or GMEM + gmate. The difference between the rates for McCoy cells in the two media were not statistically significant ( $P > 0.05$ ).

The rate of uptake of glutamate was lower than that of glutamine in all cases. However, the adaptation of McCoy cells to GMEM + gmate induced a substantial ( $\times 2$ ) and significant increase in the capacity to transport glutamate from the medium. Of considerable significance in this experiment was the finding that the rate of glutamate uptake by MDCK cells was up to an order of magnitude less than McCoy cells in either medium

## DISCUSSION

Glutamine is a major energy source and biosynthetic precursor for cell growth. It is required as a carbon source for the TCA cycle (Reitzer et al., 1979; Zielke et al., 1984) as well as a nitrogen source for the synthesis of nucleotides (Engstrom and Zetterberg, 1984) and amino acids (Levin-tow, 1957). If cells are to grow in glutamine-free media, some components must be provided to substitute for the crucial metabolic role of glutamine.

As was previously reported, some cell lines can utilise



**Fig. 6.** Profiles of cellular uptake of glutamine and glutamic acid. The rates of incorporation of (A) L-[U- $^{14}$ C]glutamine or (B) L-[U- $^{14}$ C]glutamic acid into McCoy cells grown in GMEM + gmine ( $\circ$ ), MDCK cells grown in GMEM + gmine ( $\bullet$ ) and McCoy cells adapted and grown in GMEM + gmate ( $\blacktriangle$ ) were determined over a period of 5-10 min. The protocol is described in Materials and methods.

glutamate as a substitute for glutamine (Eagle et al., 1956; Griffiths and Pirt, 1967). Either amino acid can be utilised as an intracellular energy substrate following metabolic deamination. However, the utilisation of a molar equivalent of glutamate leads to lower ammonia production. This can be beneficial for enhanced cell yields because of the potential growth inhibition of excessive concentrations of ammonia. For cells adapted to glutamine-free conditions, a combination of the utilisation of glutamate-based media and nutrient supplementation can result in a significant increase in cell yield (Hassell and Butler, 1990).

For the study reported here, two cell lines were chosen for their difference in ability to adapt to glutamate. McCoy cells adapt relatively easily. On substitution of glutamine

**Table 3. The rate of uptake of L-[U-<sup>14</sup>C]glutamine and L-[U-<sup>14</sup>C]glutamate into cells**

Cell line	Medium	Initial uptake rate (nmol/min per mg cell protein)	
		Glutamine	Glutamate
McCoy	GMEM+gmine	3.22 ± 0.59	0.250 ± 0.044*
McCoy	GMEM+gmate	4.50 ± 0.41	0.540 ± 0.095*
MDCK	GMEM+gmine	2.63 ± 0.50*	0.042 ± 0.003*

The values are means (± standard deviation) of three independent experiments. The significance of differences between values was determined by Student's *t*-test.

\* denotes values which are significantly different from the other two values for that amino acid ( $P < 0.01$ ).

for glutamate, a short lag and slow growth phase occurs but normal growth rates are assumed after 2-3 passages. However, MDCK cells show an inability to grow in a glutamate-based media. Cell division stopped immediately on substitution of glutamine for glutamate. Even in a combined glutamine/glutamate (0.5/1.5 mM) medium the growth rate was reduced to almost 50% that of controls. The primary objective of our study was to establish the metabolic changes associated with the adaptation of cells to a glutamine-free medium and to determine the reason for the difference in response between MDCK and McCoy cells.

It has been known for some time that the induction of the enzyme, glutamine synthetase is crucial to the adaptation process (Griffiths, 1973). Glutamine synthetase catalyses the formation of glutamine from glutamate and ammonia in an ATP-dependent reaction. For cells growing in a glutamate-based medium this reaction would be essential to provide the minimum level of intracellular glutamine required for the biosynthetic pathways. Glutamate would be expected to substitute for glutamine as a carbon fuel for the TCA cycle without the need for enzymatic changes.

The regulation of glutamine synthetase has been well documented. The extracellular glutamine concentration has been shown to affect the glutamine synthetase activity of cells. An increase in specific activity of the enzyme ranging from 4-fold to 10-fold in response to removal of glutamine from the culture medium has been shown for hepatoma cells (Crook and Tomkins, 1978), neuroblastoma (Lacoste et al., 1982), Chinese hamster V79 cells (Milman et al., 1975) and L6 skeletal muscle cells (Feng et al., 1990). This increase has been shown to be associated with protein synthesis but not de novo RNA synthesis (Milman et al., 1975; Sandrasagra et al., 1988). The glucocorticoid, dexamethasone, can cause an increase in enzyme activity but by a different mechanism involving stimulation of mRNA synthesis (Sandrasagra et al., 1988; Feng et al., 1990). The addition of glutamine has been reported to cause a rapid decline in enzyme activity with a  $t_{1/2}$  of activity loss of 2-4 h (Crook and Tomkins, 1978; Sandrasagra et al., 1988; Feng et al., 1990).

The pattern of changes in glutamine synthetase activity reported here for McCoy and MDCK cells in response to glutamine concentrations in the medium are compatible with previous findings. The glutamine concentration of the medium was completely depleted after 6 days of cell culture. This was associated with a 6- to 9-fold increase in

intracellular enzyme activity. The apparent Michaelis constant ( $K_m$ ) with respect to glutamate also decreased from mean values of 4.5 mM to 2.5 mM. This compares with an apparent  $K_m$  value of 3.1 mM determined for the enzyme extracted from Chinese hamster liver (Tiemeier and Milman, 1972). In our experiments, the kinetic effects of varying glutamate concentrations were monitored using a radioactive assay, which results in lower  $V_{max}$  values compared to the more convenient glutamyltransferase assay, as has been previously reported (Miller et al., 1978).

Addition of glutamine to glutamine-free cultures caused a rapid decline in glutamine synthetase activity in our experiments with a  $t_{1/2}$  of 4-5 h. Removal of glutamine from culture media caused an increase in enzyme activity which doubled in value within 5-10 h. No apparent significant differences were observed between the glutamine synthetase activities of the two cell lines during the course of these experiments. Specific activities of up to 10 nmol/min per mg protein were measured for both cell lines. However, despite the apparent similarities of glutamine synthetase levels and regulation, only McCoy cells were able to grow in glutamine-free medium with a constant glutamine synthetase activity of around 6 nmol/min per mg protein.

The dependency of a range of human lymphoblastoid cells on glutamine for growth has been shown to be related to the intracellular activity of glutamine synthetase (Kitoh et al., 1990). In this study glutamine dependency was measured by the minimum glutamine concentration required to allow cell growth at 50% of the rate of controls. The glutamine dependency showed a good inverse correlation with the glutamine synthetase activities of 13 independently grown cell lines. However, we were unable to detect a similar relationship for the McCoy and MDCK cells reported here.

We next turned our attention to membrane transport of the amino acids in order to explore differences between glutamine/glutamate metabolism of McCoy and MDCK cells, in an attempt to explain the difference in growth response to glutamine-free medium. Membrane transport has been shown to be one of the regulatory steps of glutamine metabolism (Ardawi, 1986; Ardawi and Newsholme, 1983; Jenkins et al., 1992). Thus, it would not be surprising if this were also the case for the metabolism of glutamate.

Transport systems for acidic amino acids including glutamate were reviewed by Lerner (1987). It is clear that although rapid glutamate uptake systems have been found in cells of the nervous system and some mature muscle cell lines, relatively low transport rates are observed in other cells (Baetge et al., 1979). Three independent transport systems for glutamate have been described in cultured human fibroblasts (Dall'Asta et al., 1983). One of these transport systems has been shown to undergo adaptive regulation in response to cystine (Bannai and Kitamura, 1982) or electrophilic agents which interact with intracellular glutathione (Bannai, 1984). Such adaptive responses have led to increased transport rates of up to 3-fold.

In our experiments, rates of utilisation of glutamine and glutamate were measured by radioactive assays for McCoy and MDCK cells over a period of 5 or 10 min. The measured rate of cellular incorporation of glutamine was significantly lower (nearly 20%) in MDCK cells compared

to McCoy cells. An initial rate of incorporation of 0.25 nmol glutamate/min per mg protein for McCoy cells increased to 0.54 nmol/min per mg cell protein following adaptation in glutamine-free medium. This may be an indication of an adaptive regulatory response similar to that previously described for glutamate transport in human fibroblasts (Bannai and Kitamura, 1982). The rate of glutamate transport in MDCK cells was 0.042 nmol/min per mg protein, which was an order of magnitude lower than for the McCoy cells. It would seem probable that this low rate of glutamate transport into the MDCK cells is insufficient to supply the metabolic requirements for cell growth. The difference in uptake rates is likely to account for the variable response between the two cell lines in adaptation to growth in glutamine-free medium.

Thus, we propose that despite the importance of glutamine synthetase in the adaptation of cells to glutamine-free medium, the ability to induce enhanced levels of the enzyme does not account for the growth differences between the two cell lines studied here. Rather, differences in the intrinsic ability for glutamate transport via the cell membrane may account for the growth differences in the glutamine-free media. Furthermore, the apparent adaptability of the glutamate transport system in McCoy cells enhances this difference between the two cell lines.

The presence of an adequate glutamate transport system should be an important indicator in the initial selection of cells for adaptation to glutamine-free media.

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