Adaptation to non-ammoniagenic medium and selective substrate feeding lead to enhanced yields in animal cell cultures

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Summary

Methods for the adaptation of three animal cell lines to media in which glutamine is replaced with either glutamate or 2-oxoglutarate are described. The cell lines differ in their rate of adaptation. The consequences of the adaptation of the McCoy cell line to a glutamate-based medium was measured in terms of: cell yield (increased by 17%), ammonia accumulation (reduced by 70%), glucose consumption (decreased by >70%) and lactate accumulation (decreased by >75%).

The value of such adaptation and concomitant changes in energy metabolism lies in the potential for increased cell yields. Batch feeding of adapted cells in a microcarrier culture with a cocktail of glucose and amino acids resulted in increases in cell yields of 80% compared to unfed controls.

Key words: adaptation, cell culture, energy metabolism.

Introduction

Glucose, owing to its rapid metabolism, has been assumed to be the major energy source for cultured cells (Levintow and Eagle, 1961). Under aerobic conditions, such energy could be derived directly from glucose by glycolysis to pyruvate and subsequent aerobic oxidation of the latter in the citric acid cycle.

However, several authors have suggested that very little glucose carbon enters the citric acid cycle in cultured cells (Morrell and Froesch, 1973; Reitzer *et al.* 1979). Evidence suggests that a large proportion of glutamine is utilized in aerobic energy metabolism in HeLa cells (Reitzer *et al.* 1979), human diploid fibroblasts (Zielke *et al.* 1978) and a variety of other cells (Wice *et al.* 1981).

One consequence of such metabolism is the accumulation of ammonia. Reports have linked high concentrations of ammonia with growth inhibition (Butler *et al.* 1983; Butler and Spier, 1984), although such effects may be cell line-dependent (Butler *et al.* 1990).

Whereas ammonia can be rapidly removed *in vivo*, no such mechanism exists under cell culture conditions. One method of overcoming this problem is to use alternative substrates that are relatively non-ammoniagenic compared to glutamine.

Many culture media formulations currently in use are modifications of the original formulation reported by Eagle (1959) and contain glutamine at relatively high concentrations (2–4 mM) compared to other amino acids. In Eagle's early studies on essential medium components, he described unsuccessful attempts to substitute glutamine with either glutamate or 2-oxoglutarate for growth of a mouse fibroblast cell line and the HeLa cell line (Eagle, 1955). However, in a later study he described the successful use of high concentrations (20 mM) of glutamate as a substitute for glutamine for several unspecified cell lines (Eagle, 1959).

Griffiths and Pirt (1967) developed further a protocol based on that of Paul and Fottrell (1963) whereby cells could be grown in a medium without added glutamine. The basis of this protocol was to transfer cells to a medium containing a high concentration (20 mM) of glutamate, which was gradually reduced in concentration at subsequent passages.

In experimental work reported here, this protocol was modified to adapt BHK, McCoy and Vero cells to either glutamate- or 2-oxoglutarate-supplemented culture medium based on GMEM with 10% serum and 25 mM glucose. In such media, glutamate or 2-oxoglutarate was eventually substituted for glutamine on a mole for mole basis.

The ammonia accumulation concomitant with altered medium composition was assessed for each of the three adapted cell lines. McCoy cells were most readily adapted to glutamine-free media and were selected for studies into the metabolic consequences of adaptation. These cells showed a moderate sensitivity to 2 mM ammonia (50–60% reduction in cell yield) compared to other cell lines (Butler *et al.* 1990). The cells were grown in microcarrier culture to remove the restriction to growth imposed by the surface area limitations of T-flask culture. This proved an ideal system for examining the effects of batch feeding of the cultures with a nutrient cocktail.

Materials and methods

Culture medium

The standard growth medium used throughout these studies was the Glasgow modification of Eagle's medium (GMEM; Stoker and

MacPherson, 1964), supplemented with 10% bovine serum (v/v). This was supplemented with glucose at 25 mM and either glutamine, glutamate or 2-oxoglutarate. These media formulations are described as: GMEM-Gln, GMEM containing glutamine at 4 mM; GMEM-Glu, GMEM in which glutamine was replaced by 4 mM glutamate; GMEM-2-oxo, GMEM in which glutamine was replaced by 4 mM 2-oxoglutarate.

Cultures

T-flasks. Cells were inoculated into 10 ml of culture medium in 25 cm^2 T-flasks or 50 ml in 150 cm^2 T-flasks.

Microcarrier cultures. Cells were inoculated at 2×10^5 cells ml^{-1} into 100 ml of culture medium, containing microcarriers (Cytodex 1 from Pharmacia) at 5 mg ml^{-1}. Spinner flasks (Bellco) were placed on a stirrer platform (Bellco), inside a CO_2 incubator (Heraeus), and maintained at a constant stirring rate of 50 revs min^{-1}. All cultures were incubated at 37°C with a 10% CO_2 overlay.

Cell lines

The following cell lines were used in this study: *Vero* are kidney fibroblasts originally from an African green monkey (Yasumara and Kawakita, 1963). They were kindly provided by Mr A. Bailey of Booth Hall Childrens Hospital, Manchester. *BHK21* are fibroblasts originally derived from Baby Golden Hamster kidneys (Stoker and MacPherson, 1964). They were kindly provided by Mr D. Coupes of Manchester University Medical School. *McCoy* are mouse LS fibroblasts (Hsu *et al.* 1957). They were supplied by Flow Laboratories, UK.

The cells were routinely tested for mycoplasma by Mr D. Coupes in the Virology laboratories at the Manchester University Medical School. All cell lines tested were negative.

Cell harvest

Cells were harvested from T-flasks by the addition of 2 ml of 0.25% trypsin in PBS and incubating for 10 min at 37°C. For Vero the further step of vigorous shaking or tapping was necessary to detach cells from the surface.

Cell enumeration

Viable cell counts were determined by the Trypan Blue dye exclusion method (Patterson, 1979). Equal volumes of cell suspension and the Trypan Blue reagent (0.2%, v/v, Trypan Blue in PBS) were mixed and the unstained cells were counted using a haemocytometer. Total cell counts were determined by the method of Sanford *et al.* (1951). After a 2 h incubation period at 37°C in a solution of 0.1 M citric acid and 0.1% Crystal Violet, the stained nuclei from the lysed cells were counted using a haemocytometer.

Adaptation protocol for BHK and Vero cells to GMEM-Glu and GMEM-2-oxo

Cells were grown in medium containing 4 mM glutamine to about 80% of confluence, the spent medium was replaced with modified medium (GMEM with the omission of glutamine and substitution with glutamate or 2-oxoglutarate at a concentration of 20 mM). After the cells had shown an increase of about 10% in number, they were twice sub-cultured at ratios of 1:2 and 1:3 and grown in the same medium. On reaching confluence, the cells were then twice sub-cultured at a ratio of 1:4 into medium formulated to contain 15 mM glutamate or 2-oxoglutarate. At confluence, cells were sub-cultured at a 1:4 ratio into medium containing 10 mM glutamate or 2-oxoglutarate. Finally, at confluence, cells were then sub-cultured into medium containing 4 mM glutamate (GMEM-Glu) or 4 mM 2-oxoglutarate (GMEM-2-oxo).

Adaptation protocol for McCoy cells to GMEM-Glu and GMEM-2-oxo

Cells were grown in standard culture medium containing glutamine at 4 mM. At confluence the cells were sub-cultured three times at a 1:4 split ratio into glutamine-free media containing either glutamate or 2-oxoglutarate at 4 mM. From this point adapted cells were passaged at similar split ratios to their glutamine-grown counterparts.

Table 1. *Complex supplement*

Component	Concentration (mM)	Concentration increase in culture (mM)
L-arginine	2.0	0.1
L-cystine (disodium)	1.0	0.05
L-glutamine*	40.0	2.0
L-glutamate*	40.0	2.0
L-histidine (HCl)	1.0	0.05
L-isoleucine	4.0	0.2
L-leucine	4.0	0.2
L-lysine (HCl)	5.0	0.25
L-methionine	1.0	0.05
L-phenylalanine	2.0	0.1
L-threonine	4.0	0.2
L-tryptophan	0.4	0.02
L-tyrosine (disodium)	2.0	0.1
L-valine	4.0	0.2
$NaHCO_3$	250.0	12.5
D-glucose	250.0	12.5

*Glutamine was present in the supplement for GMEM-Gln and glutamate for GMEM-Glu.

Feeding experiments

Simple supplements. Glutamine or glutamate. From day 2 of the culture period, batch addition of 2 ml of a concentrate of either glutamine or glutamate was commenced so that the culture concentration of these amino acids was increased by 2 mM.

Complex supplement. Amino acid mixture. At daily intervals from day 2, 5 ml of the mixture described in Table 1 was added to the relevant microcarrier cultures. This mixture represents 50% of the original GMEM concentration of each amino acid and glucose.

Ammonia assay

Ammonia concentrations were measured with an ammonia gas-detecting electrode (Orion model 95-10), connected to a mV meter (Orion). Samples (5 ml) were made basic by the addition of 0.2 ml NaOH (10 M). Each tenfold increase in concentration of ammonia gave rise to a 59 mV change in electrode potential. This corresponds to the theoretical change associated with the Nernst equation. A standard curve was produced using ammonium chloride at 100 mM, 10 mM and 1 mM.

Glutamine and glutamate analysis

Medium samples were derivatized with phenylisothiocyanate (PITC) then analysed by High Performance Liquid Chromatography (HPLC) using a modification of the method described by Cohen *et al.* (1986).

2-Oxoglutarate analysis

2-Oxoglutarate was measured by the oxidation of NADH in the presence of glutamate dehydrogenase (GDH). This spectrophotometric assay was modified from the method described by Bergmeyer and Bernt (1963) and uses the following reaction:

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NADH} \overset{\text{GDH}}{\rightleftharpoons} \text{glutamate} + H_2O + NAD^+$$

A 25 µl portion of 2-oxoglutarate (25–250 µM) or the sample was added to a mixture of 25 µl NADH (6 mg ml^{-1} in $NaHCO_3$, 10%, w/v), 10 µl glutamate dehydrogenase (supplied as a suspension in 2 M $(NH_4)_2SO_4$, Sigma) and 2 ml KH_2PO_4 (0.2 M at pH 6.8 with NaOH). This was incubated at 20°C for 5 min and the absorbance at 340 nm was measured. The concentration of 2-oxoglutarate in the sample was calculated by comparison with standards. A linear response was found between the absorbance at 340 nm and the concentration of 2-oxoglutarate in the range 25 µM to 250 µM.

Glucose and lactate

Both glucose and lactate were measured using a YSI industrial analyser (Clandon Scientific), a system based on specific enzymes immobilized in membranes.

Results

Adaptation of BHK21 cells to glutamine-free media

The change in mean doubling time over the 15- to 20-day period of adaptation of BHK cells to glutamine-free media is shown in Fig. 1. After a period of 15 days the cells grown in GMEM-Glu and GMEM-2-oxo had similar doubling times to cells grown in GMEM-Gln. Cells appeared to adapt more readily to GMEM-Glu than to GMEM-2-oxo. On each subsequent sub-culture there was no further change in mean doubling times.

Adaptation of McCoy cells to glutamine-free media

The change in mean doubling time over the adaptation period to glutamine-free media for McCoy cells is shown in Fig. 2. Within 10 days both series of cells cultured in glutamine-substituted media had mean doubling times similar to their glutamine-grown counterparts, i.e. 19–24 h. Cells appeared to adapt more readily to GMEM-Glu than to GMEM-2-oxo.

Adaptation of Vero cells to glutamine-free media

The change in mean doubling time over the 30-day period of adaptation of Vero cells to glutamine-free media is shown in Fig. 3. Initially, relatively long mean doubling times were observed for both GMEM-Glu and GMEM-2-oxo. These doubling times gradually decreased during the adaptation period. After 30 days of culture under these conditions, no further changes in doubling times were observed.

By the end of this 30-day period the GMEM-Glu-cultured Vero cells had achieved mean doubling times that were similar to their glutamine-grown counterparts with typical doubling times of 20–25 h. However, those cells in GMEM-2-oxo had a doubling time of 28–33 h. This longer doubling time did not change, irrespective of the number of subsequent passages in GMEM-2-oxo.

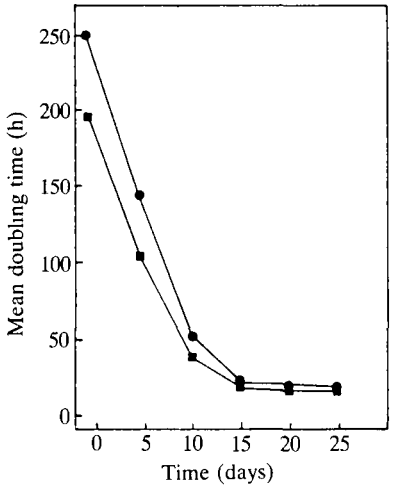

Fig. 1. Change in mean doubling time of BHK21 cells in glutamine-free medium. At time 0 the GMEM-Gln culture medium was replaced with glutamine-free medium. BHK cells were then cultured in GMEM-2-oxo (●) or in GMEM-Glu (■). In glutamine-based medium these cells had a doubling time of 22–25 h.

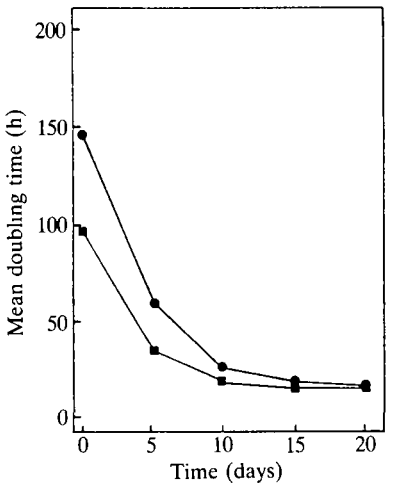

Fig. 2. Change in mean doubling time of McCoy cells in glutamine-free medium. At time 0 the GMEM-Gln culture medium was replaced with glutamine-free medium. McCoy cells were then cultured in GMEM-2-oxo (●) or in GMEM-Glu (■). In glutamine-based medium these cells had a doubling time of 19–24 h.

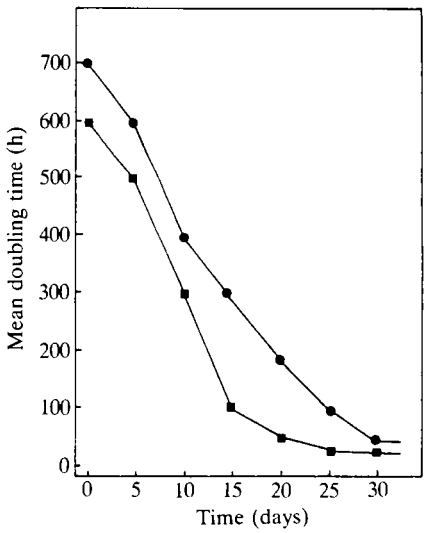

Fig. 3. Change in mean doubling time of Vero cells in glutamine-free medium. At time 0 the GMEM-Gln culture medium was replaced with glutamine-free medium. Vero cells were then cultured in GMEM-2-oxo (●) or in GMEM-Glu (■). In glutamine-based medium these cells had a doubling time of 20–25 h.

Ammonia production

BHK, Vero and McCoy cells adapted to growth in glutamine-free media were inoculated into 10 ml cultures in 25 cm^2 T-flasks. After 3 days of incubation the cells were counted and the culture media assayed for ammonia.

Final ammonia concentrations were reduced by 55 % to 65 % in individual cultures grown in glutamine-free media compared to those in glutamine-containing media (Table 2).

Cell inocula were the same for all cultures at 1.5×10^5

Table 2. *Final ammonia concentration of culture medium for three adapted cell lines in glutamine-free media*

Cell line	Ammonia concentration (mM)		
	GMEM-Gln	GMEM-Glu	GMEM-2-oxo
Vero	2.46	1.01	0.97
McCoy	2.14	0.99	0.89
BHK	2.11	0.88	0.77

Values are means, where $n=3$. Standard deviation was less than 10% of the mean in all cases.

cells. Final cell yields were 5×10^5 for the McCoy cells cultured in GMEM-Gln, GMEM-Glu and GMEM-2-oxo. BHK cell yields were 3.6×10^5 for each of the three culture media. In Vero cell cultures the yield of 4.6×10^5 cells in GMEM-Gln was 10% greater than in GMEM-Glu and 25% greater than in GMEM-2-oxo.

Microcarrier cultures

Microcarrier cultures offer a much greater surface area for growth than T-flasks. At a microcarrier concentration of $5\,mg\,ml^{-1}$ the surface area for growth is $30\,cm^2\,ml^{-1}$ and in batch culture this is unlikely to limit cell yield. The cell yields observed in batch microcarrier cultures of McCoy cells in GMEM-Gln, GMEM-Glu and GMEM-2-oxo are shown in Fig. 4. The GMEM-Gln cultures had maximum concentrations of 1.6×10^6 to 1.8×10^6 cells ml^{-1} after 5 days. Maximum cell yields were nearly 17% greater for the GMEM-Glu cultures at between 1.85×10^6 and 2.1×10^6 cells ml^{-1} after 4 days. Maximum cell yields for the GMEM-2-oxo cultures were 6×10^5 cells ml^{-1} after 4 days.

Changes in substrate and ammonia concentrations were measured for the cultures described in Fig. 4 and are shown in Fig. 5. The amino acids, glutamine and glutamate, were reduced to near zero concentration, whereas the concentration of 2-oxoglutarate decreased by 1.5 mM. Lower concentrations of accumulated ammonia were measured in GMEM-Glu and GMEM-2-oxo cultures compared to GMEM-Gln. The ammonia concentration increased to a maximum of 3.7 mM for GMEM-Gln cultures, whereas the ammonia concentration of the GMEM-Glu cultures remained less than 1.1 mM and 1 mM for GMEM-2-oxo. The initial ammonia concentration was 0.45 mM for all cultures.

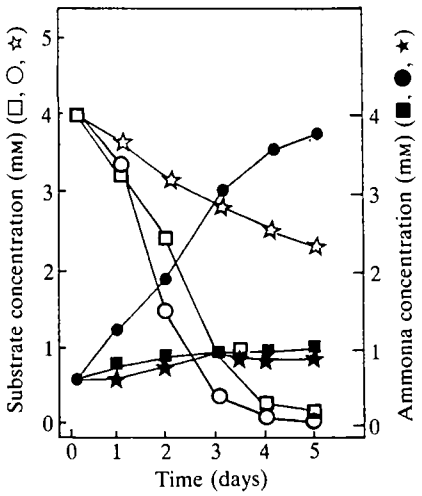

Fig. 5. Changes in glutamine, glutamate, 2-oxoglutarate and ammonia concentrations for microcarrier cultures of McCoy cells. Glutamine, glutamate, 2-oxoglutarate (open symbols) and ammonia concentrations (filled symbols) for microcarrier cultures of McCoy cells in GMEM-Gln (○), GMEM-Glu (□) and GMEM-2-oxo (☆). Cultures were at 2.5×10^5 cells ml^{-1} and incubated at 37°C with a 10% CO_2 overlay.

Changes in glucose and lactate concentrations in the microcarrier cultures are shown in Fig. 6. Glucose utilization was greatest in the GMEM-Gln cultures with a decrease in medium concentration of 17 mM. In these cultures the lactate content of the medium increased to 20 mM. In the GMEM-Glu and GMEM-2-oxo cultures the glucose utilization was relatively low, changing by 5 mM and 3 mM, respectively, over the same period with the lactate increasing to 4 mM.

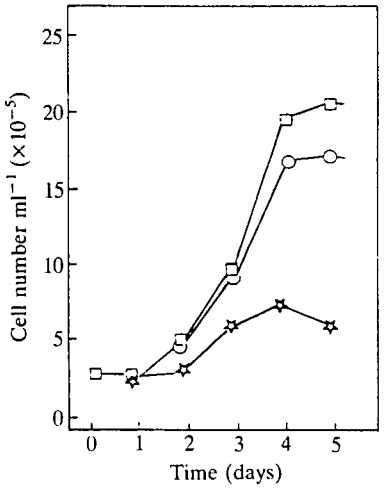

Fig. 4. McCoy cell growth in microcarrier cultures in glutamine containing and glutamine-free medium. Cell yields of microcarrier cultures in GMEM-Gln (○), GMEM-Glu (□) or GMEM-2-oxo (☆) media for McCoy cells inoculated at 2.5×10^5 cells ml^{-1} and incubated at 37°C with a 10% CO_2 overlay ($n=2$).

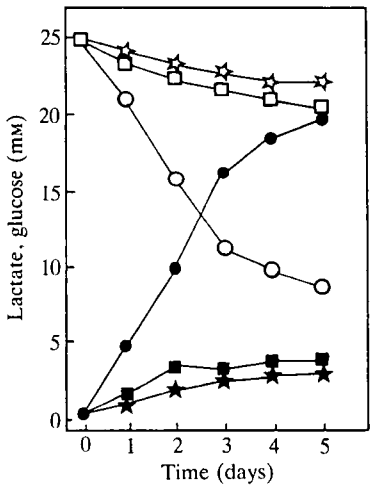

Fig. 6. Glucose utilization and lactate production by McCoy cells in microcarrier culture supplemented with glutamine, glutamate or 2-oxoglutarate. Glucose (open symbols) and lactate (filled) for microcarrier cultures of McCoy cells in GMEM-Gln (○,●), GMEM-Glu (□,■) and GMEM-2-oxo (☆,★). Cultures were at 2.5×10^5 cells ml^{-1} and incubated at 37°C with a 10% CO_2 overlay.

Table 3. *Growth parameters related to microcarrier cultures of McCoy cells*

Culture	Growth yields (cells pmol^{-1})						Product yields (mol mol^{-1})	
	Glucose	Gln	Glu	2-oxo	NH_3	Lactate	Lac/Hex	NH_3/a.a.
GMEM-Gln	0.08	0.36	–	–	−0.45	−0.07	1.15	0.80
GMEM-Glu	0.34	–	0.43	–	−2.74	−0.58	0.59	0.16
GMEM-2-oxo	0.12	–	–	0.23	−0.73	−0.14	0.85	0.32

The values of growth and product yields were calculated from data in Figs 4–6, which describe microcarrier cultures of McCoy cells. Hex, hexose; Lac, lactate; a.a., amino acid.

Growth yields

From the results presented in Figs 4, 5 and 6, growth yield values were calculated from the equation:

$$Y = X/S,$$

where X is the increase in cell concentration resulting from the utilization of S moles of substrate. This equation has been used to produce growth yield data from the microcarrier cultures of McCoy cells in GMEM-Gln, GMEM-Glu and GMEM-2-oxo and the results are presented in Table 3. The total increase in cell concentration and total substrate utilization throughout the culture period have been used in each calculation.

The growth yields were significantly higher in the GMEM-Glu cultures with respect to both glucose and glutamate compared to the equivalent substrates in GMEM-Gln and GMEM-2-oxo. The lactate:hexose ratio and ammonia:amino acid ratio were calculated for each of the three culture conditions as values for the entire culture period and are shown in Table 3. Low lactate and ammonia yields with respect to substrate utilization occurred in the GMEM-Glu cultures.

Single amino acid feeding

The amino acids, glutamine or glutamate were added to cultures on a daily basis from day 2 onwards. Each amino acid was added as a 5 ml bolus, thereby increasing its concentration in the medium by 1 mM. Growth curves presented in Fig. 7 are the means of two independent cultures and show that the effect of such selective feeding was to increase the cell concentration by 13 % in GMEM-Gln to a maximum of 1.9×10^6 cells ml^{-1} and by 25 % in GMEM-Glu to a maximum of 2.4×10^6 cells ml^{-1} compared to their respective unfed counterparts.

Ammonia accumulation was greater in the GMEM-Gln-fed cultures with a maximum concentration >5 mM compared to a maximum of only 1.5 mM in the GMEM-Glu-fed cultures.

Glucose utilization and lactate accumulation for these batch-fed cultures are shown in Fig. 8. Glucose consumption was greater for GMEM-Gln than for the GMEM-Glu cultures. Similarly, the lactate accumulation for the GMEM-Gln cultures (>20 mM) was more than four times that of the GMEM-Glu cultures (<5 mM).

Complex feeding

The effects of adding an amino acid and glucose mixture on the final cell yield of microcarrier cultures of McCoy cells in GMEM-Gln and GMEM-Glu were investigated.

The mixture of amino acids and glucose described in Table 1 was added on a daily basis after 2 days. The addition of this concentrate increased the concentration of each component by 50 % of its original value in the culture medium. The effect of feeding the cultures with this mixture was to increase the final cell yield for GMEM-Gln cultures to 2.6×10^6 cells ml^{-1} and GMEM-Glu cultures to 3.0×10^6 cells ml^{-1} (Fig. 9). Final cell concentrations for the GMEM-Glu-fed cultures were nearly double the maximum observed for unfed GMEM-Gln cultures.

Glucose utilization and lactate accumulation for these

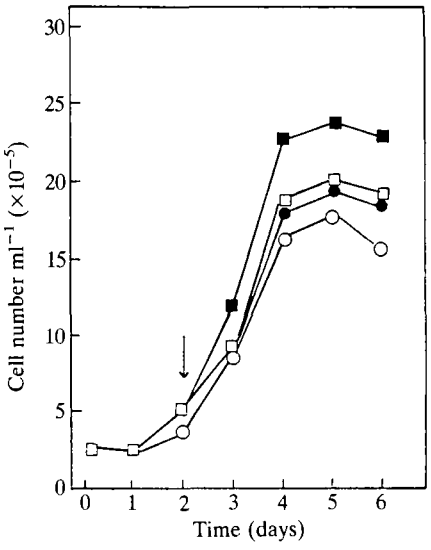

Fig. 7. Growth of McCoy cells in microcarrier cultures with daily supplements of single amino acids. Cell concentrations for microcarrier cultures of McCoy cells in GMEM-Gln with (●) and without (○) feeding of glutamine, and GMEM-Glu with (■) and without (□) feeding of glutamate. Daily feeding was from day 2 onwards (n=2).

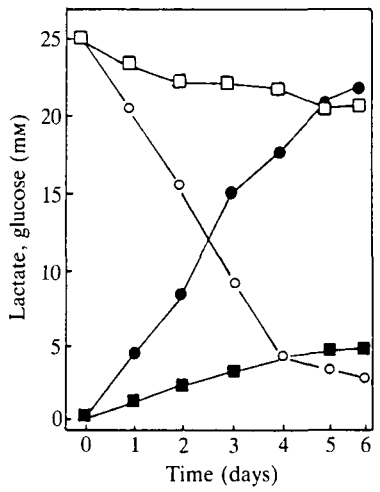

Fig. 8. Glucose utilization and lactate production by McCoy cells in microcarrier cultures fed with glutamine or glutamate. Glucose (open) and lactate (filled) concentrations of microcarrier cultures of McCoy cells in GMEM-Gln (○), and GMEM-Gln (□) with daily feeding of glutamine or glutamate from day 2 onwards (n=2).

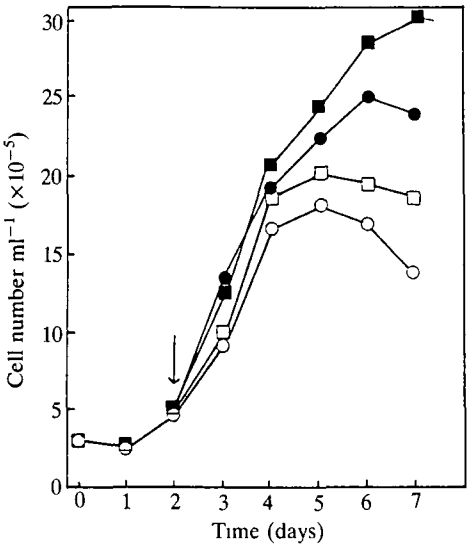

Fig. 9. Growth of McCoy cells in microcarrier cultures with daily additions of complex supplement. Cell concentrations of microcarrier cultures of McCoy cells in GMEM-Gln with (●) and without (○) feeding of the glutamine-based mixture and GMEM-Glu with (■) and without (□) feeding of the glutamate-based mixture. Daily feeding was from day 2 onwards (n=2).

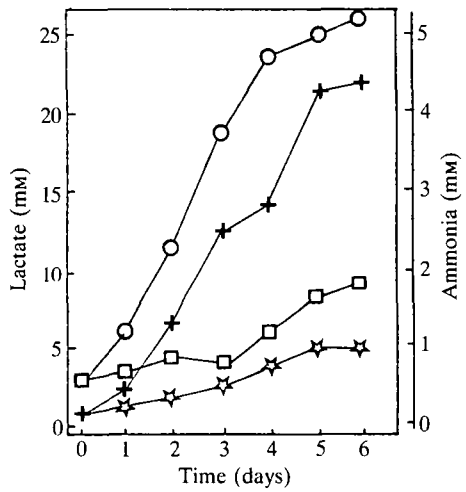

Fig. 10. Ammonia and lactate accumulation in microcarrier cultures of McCoy cells with daily addition of complex supplement. Ammonia concentrations for GMEM-Gln (○) and GMEM-Glu (□), and lactate concentrations for GMEM-Gln (+) and GMEM-Glu (☆) cultures, fed from day 2 onwards with glutamine- and glutamate-based supplements, respectively (n=2).

fed cultures are shown in Fig. 10. As in the earlier observations, the glucose consumption was much greater for the GMEM-Gln cultures than for the GMEM-Glu cultures, and lactate accumulation for the GMEM-Gln cultures (at a maximum >20 mM) was more than four times greater than that for the GMEM-Glu cultures (<5 mM). The ammonia production was significantly higher for these fed cultures and might be explained by the de-amination of the added amino acids.

Discussion

The primary objective of the work reported here was to assess the potential for using relatively non-ammoniagenic media for growing cell lines in culture. Three cell lines were selected to assess their ability to adapt to non-ammoniagenic media. Two experimental protocols are described for the adaptation of the cells to media containing glutamine analogues. The first protocol involved a mole to mole substitution of glutamine with glutamate or 2-oxoglutarate. This was successful for the McCoy cells, which attained normal growth rates in 5–10 days.

A more complex protocol was required for the adaptation of BHK and Vero cells and was based on that described by Griffiths and Pirt (1967). In this protocol a high initial concentration of the glutamine analogue was gradually reduced with time. After a period of adaptation, doubling times similar to those of the glutamine-grown cells were established for the BHK and Vero cells within 15–30 days. In all cases, cells more readily adapted to the glutamine-free media containing glutamate. The nature of the adaptation process is unknown but is likely to be due to alterations in the metabolic profile of the cells arising from changes in enzyme activities or altered substrate-uptake rates. The possibility of cell selection can be eliminated because cells were observed to grow continuously on the substratum provided.

The growth of several cell lines, including BHK and McCoy, was adversely affected by the presence of ammonia when added at concentrations >0.5 mM to the culture medium (Butler *et al.* 1990). The contention that a lowering of ammonia concentration in culture media would result in increased cell yields for ammonia-sensitive cell lines is supported by studies with perfusion cultures (Butler *et al.* 1983; Nahapetian *et al.* 1986). Furthermore, enhanced cell yields of >50% have been reported for a myeloma cell line when ammonia concentrations in the culture medium were reduced by the use of an absorbent (Iio *et al.* 1984).

For each of the three cell lines grown in T-flasks, ammonia accumulation was considerably reduced in glutamine-free media. The reductions were such that only 35% to 45% of the ammonia concentration observed in GMEM-Gln medium accumulated in the glutamine-free media. A 9% lower ammonia concentration was observed for those cells cultured in GMEM-2-oxo, compared to those cultured in GMEM-Glu (Table 2).

The potential for enhanced growth yields in the non-ammoniagenic media was investigated in microcarrier cultures. The microcarrier concentration used offered a considerably greater surface area (30 cm^2 ml^{-1} of medium) for the growth of the anchorage-dependent cells compared to T-flasks (3 cm^2 ml^{-1} of medium) (Butler and Thilly, 1982).

The microcarrier cultures of McCoy cells produced a maximum ammonia concentration of nearly 4 mM in GMEM-Gln but only about 25% of this value in GMEM-Glu (0.98 mM) and GMEM-2-oxo (0.87 mM) cultures. Cell yields were markedly reduced for GMEM-2-oxo compared to GMEM-Gln cultures, a difference that was not apparent in T-flasks. However, cell yields were increased by about 17% in GMEM-Glu cultures compared to GMEM-Gln. Such enhanced cell yields offer considerable advantage in large-scale culture technology.

An additional benefit of the use of glutamine analogues is that they are heat-stable, an advantage that has been previously recognised (Nagle and Brown, 1971; Mizrahi and Avihoo, 1976; Keay, 1977). Thus the glutamine-free medium may be autoclavable and may be stored at ambient temperatures without problems of decomposition.

This is particularly important for use in large-scale processes.

The results of cell growth on microcarriers suggest that glucose consumption is closely related to the presence of other substrates; glutamine, glutamate or 2-oxoglutarate, and suggest that high initial glutamine concentrations contribute to high rates of glycolysis. Some evidence exists to support this contention: Ardawi and Newsholme (1983) showed that when both glucose and glutamine were presented to rat lymphocytes the rates of utilization of both substrates increased significantly. They suggested that this was, in part, due to enhanced flux through glycolysis brought about by the activation of 6-phosphofructokinase.

The enhanced cell yield in GMEM-Glu may be related to the reduction in ammonia accumulation in these cultures, which allows the energy metabolism of the available substrates to proceed more efficiently. Growth yields for glucose and amino acids are presented in Table 3. Glucose utilization in the GMEM-Glu cultures is markedly more efficient than in the GMEM-Gln cultures. This is reflected by an increase in growth yield of $\times 4.25$ and a considerably lower production of lactate per mole of glucose utilized. Similarly, an increased efficiency of about 16 % can be seen for glutamate utilization compared to glutamine. These data are supported by previous work (Griffiths and Pirt, 1967), which showed enhanced growth yields of mouse L cells in relation to the essential amino acids in glutamate-based media.

We propose that the lower growth yields in GMEM-Gln may be a consequence of the greater ammonia production in these cultures. Ammonia may cause the depletion of the α-keto acid metabolites of glycolysis and the tricarboxylic acid cycle, such as pyruvate and oxaloacetate. This could explain the observed increase in the concentration of selected amino acids such as alanine and aspartate in the medium (Butler *et al.* 1983; Lanks and Li, 1988; Miller *et al.* 1989). The depletion of such intracellular metabolites would cause a disturbance to energy metabolism, resulting in an increased requirement for carbon substrates such as glucose and glutamine. Furthermore, the lower lactate:hexose ratios observed for the GMEM-Glu and GMEM-2-oxo cultures may be indicative of a more efficient breakdown of glucose, because the resulting metabolic intermediates are not required for the detoxification of accumulated ammonia as in GMEM-Gln.

In batch culture glutamine and glutamate reached near zero concentration after 5 days. Such nutrient depletion resulted in a cessation of growth, which could be alleviated by batch feeding. The results of the addition of either glutamine or glutamate to their respective GMEM-Gln and GMEM-Glu cultures showed an increase in cell yield for both cultures. The final cell yield was greater for the GMEM-Glu cultures, with a maximum value 20 % greater than the GMEM-Gln cultures.

The possibility that other substrates could become growth-limiting was investigated in the second of the feeding experiments in which a mixture of amino acids and glucose was fed into the cultures. Maximum cell densities were higher for both fed cultures in comparison to their unfed counterparts: a cell density of 3×10^6 cells ml^{-1} was attained in the GMEM-Glu cultures.

These experiments indicate that a sequential series of parameters limit cell yield in batch culture. We have identified two such parameters: ammonia accumulation and availability of carbon substrates. Thus, a regime of selective batch feeding in non-ammoniagenic cultures can offer significantly higher cell yields. These results will be particularly valuable for the commerical production of biological products from large-scale animal cell cultures.

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