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HIGH YIELDS FROM MICROCARRIER CULTURES BY MEDIUM PERFUSION

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SUMMARY

A culture perfusion system is described for the growth of anchorage-dependent cells on microcarriers. The critical component of this system is a column separator, which removes medium while allowing the microcarriers to remain in the culture. Amino acids and ammonia were analysed during cell growth of the perfusion culture. None of the amino acids was completely utilized. The accumulation of ammonia to 2·3 mM was observed and may be responsible for, or coincident with, events limiting further cell growth. It is suggested that oxygen deprivation and growth inhibitor accumulation, rather than nutrient depletion, are the major factors in limiting even higher cell yields.

INTRODUCTION

Significant progress has been made in the culture of anchorage-dependent animal cells (van Wezel, 1967, 1973) by the discovery of low-charge microcarriers, which serve as an anchor for cell growth in suspension (Levine, Wong, Wang & Thilly, 1977; Levine, Wang & Thilly, 1979; Thilly & Levine, 1979). Microcarrier cultures have been used to obtain relatively high yields of cells, which can be used for viral production (Giard, Thilly, Wang & Levine, 1977) or interferon production (Giard *et al.* 1979).

In order to improve cell yields even further, and to study the causes for cell growth or inhibition, we have investigated the use of a perfusion system to provide a continuous supply of medium through the microcarrier cultures. This approach was used successfully by Kruse and coworkers (Kruse, Myhr, Johnson & White, 1963; Kruse & Miedema, 1965; Kruse, Miedema & Carter, 1967; Kruse, Whittle & Miedema, 1969; Kruse, Keen & Whittle, 1970) for the growth of various cell lines in T-flasks and roller bottles. In this work we use the word perfusion as defined by Kruse *et al.* (1963, 1965, 1967, 1969, 1970) to mean a constant supply of medium through our cultures. However, various other terms have been used in the literature for similar processes: superfusion (Smith & Vale, 1980), perifusion (Gebhardt & Mecke, 1979) and circumfusion (Rose *et al.* 1970).

The value of such a perfusion system is to allow a constant supply of nutrients and

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constant removal of inhibitors that may accumulate in the culture medium. In this manner a perfusion system more closely resembles an *in vivo* situation, in which nutrients and inhibitors remain at more constant levels instead of undergoing respective depletion and accumulation, as in a batch culture system. In one set of experiments, amino acid consumption and ammonia production were monitored. In another set of experiments, perfusion of cultures with diluted medium was used to distinguish between the effects of nutrient depletion and inhibitor accumulation on cell yield. By diluting the perfusing medium, the rate of nutrient supply to the culture was lowered while the rate of removal of inhibitors was kept constant.

MATERIALS AND METHODS

Cells

Madin-Darby canine kidney (MDCK) cells were originally obtained as an established cell line derived from the epithelium of a male Cocker Spaniel in 1958 (American Type Culture Collection). We obtained MDCK cells from Flow Laboratories, McLean, VA. Routine tests by fluorescence staining (Russell, Newman & Williamson, 1975) indicated that these cells were free of mycoplasma contamination (Bioassay Systems Corp., Woburn, MA).

The cells were maintained on 150 cm^2 T-flasks in 50 ml medium or on 490 cm^2 roller bottles in 100 ml medium. Cells required for inoculation into spinner bottle cultures were harvested at the day of confluence. The medium from each T-flask or roller bottle was removed, the cells were washed in phosphate-buffered saline (PBS), and removal of cells from the plastic surface was effected by treatment with 5 ml of 1 % trypsin (Flow Labs) plus 0·2 % EDTA (Aldrich Chem. Co. Inc., Milwaukee, MI) in PBS. After treatment for 10 min at 37 °C, trypsinization was halted by addition of 8 ml of medium. The cells were centrifuged and resuspended in fresh medium. Approximately 2×10^7 cells were obtained from each 150 cm^2 T-flask and 8×10^7 cells from each roller bottle.

Medium

The medium used for all cultures was Dulbecco's modified Eagle's medium (DMEM) containing 4 mM-glutamine (Flow Labs). This was stored at −20 °C until required so that the decomposition of glutamine was minimized. The glucose was replaced by 20 mM-fructose (Sigma Chemical Co., St Louis, MO) in order to maintain a stable pH in cell cultures (Imamura, Crespi, Thilly & Brunengraber, 1982). The medium was supplemented with 10 % horse serum (Flow Labs), and the same lot of undialysed horse serum was used in all experiments.

In one set of experiments the DMEM was diluted with PBS or with a 'DMEM salt solution'. The DMEM salt solution contained the major inorganic salts of DMEM: $CaCl_2{\cdot}2H_2O$ (1·8 mM); KCl (5·4 mM); $MgSO_4{\cdot}7H_2O$ (0·8 mM); NaCl (109 mM); $NaH_2PO_4{\cdot}2H_2O$ (1·0 mM); and $NaHCO_3$ (44 mM). An independently prepared solution of $NaHCO_3$ was filter-sterilized and added to an autoclaved mixture of the other salts. The pH of the combined solution was then adjusted to pH 7·5.

Spinner cultures and microcarriers

Cells were grown in microcarrier cultures at 37 °C in a 10 % CO_2/air overlay. Two-litre spinner flasks (Wheaton Scientific Co., Millville, NJ) were used for 500 ml perfusion cultures. Smaller spinner flasks (Wilbur Scientific, Boston, MA), 250 ml and 500 ml, were used for 100 ml and 200 ml cultures, respectively. All spinner flasks were siliconized (Prosil-28, PRC Research Chemicals, Inc., Gainesville, FL).

Each culture was stirred continuously by a teflon-coated suspended magnetic stir bar (4·5 cm) maintained at 60 rev./min by means of a rotating magnetic base (Bellco Glass, Vineland, NJ). The microcarriers (Superbeads from Flow Labs) were washed in the culture medium for 10 min and added to the cultures at a concentration of 7·5 mg/ml.

Perfusion system

The system used is shown in Fig. 1. The inflow of medium to the culture was maintained by a peristaltic pump (model 7014, Cole-Palmer Instrument Co., Chicago, IL) at a rate of 1 ml/min for 500 ml cultures or 0·4 ml/min for 200 ml cultures. A silicon inflow tube (0·2 cm inner diam.; 0·5 cm outer diam.) passed from the medium reservoir through the peristaltic pump and into the culture.

The outflow from the culture was controlled by a column separator at the liquid surface. This separator consisted of a thick piece of silicon tubing, the opening of which was held at the surface of the culture. The dimensions of the separator were 10 cm length and 2·5 cm i.d. for 500 ml cultures and 10 cm × 1·3 cm for 200 ml cultures. The separator was connected to a narrow tube (0·2 cm i.d.), which carried the effluent through the pump and into a second reservoir. The outflow pump was maintained at a pumping rate of up to twice the inflow pump. The presence of the column separator resulted in progress of the culture through its length sufficiently slow for the sedimentation velocity of the microcarriers to be greater than the medium effluent velocity. The length of this column was sufficient to ensure complete separation of the microcarriers from the medium, so that the microcarriers remained in culture while the medium was continuously withdrawn.

A silicon sampling tube was inserted into the culture so that samples could be taken without disturbing the culture in the incubator. The system was autoclaved in three separate units: culture flask, inflow system and outflow system. These were assembled aseptically before use.

Medium perfusion was started 6 h after inoculation of the culture with cells. During this initial period the cells were observed microscopically to attach to the microcarriers.

Culture sampling

Samples were withdrawn from perfusion cultures via a 10 ml syringe, which was connected to the sampling tube by a plastic adapter. The first 3 ml was discarded and the second 3 ml was immediately

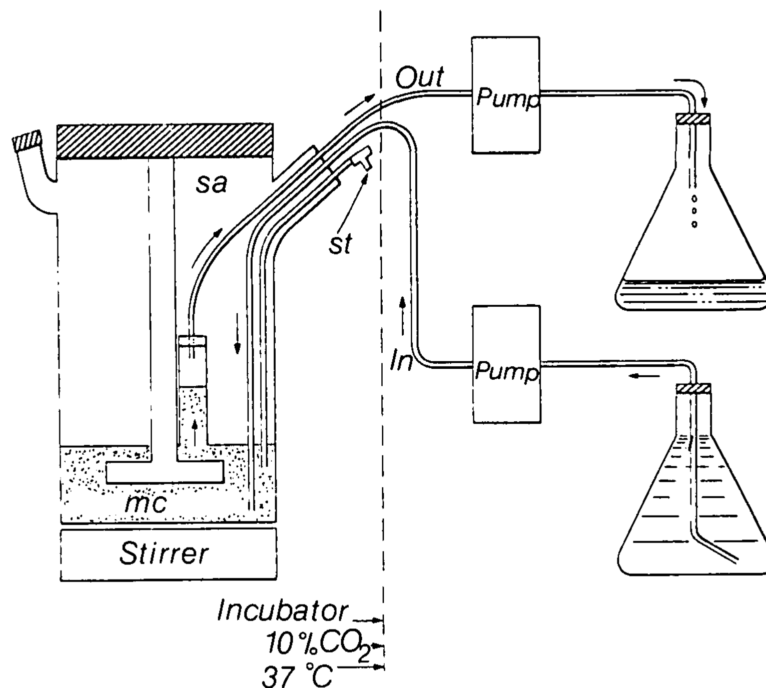

Fig. 1. Perfusion apparatus for microcarrier cultures. *sa*, separation apparatus; *mc*, microcarrier culture; *Out*, *In*, outflow and inflow; *st*, sampling tube.

centrifuged at low speed. The supernatant was deproteinized with perchloric acid (3 %) and stored at −20 °C before medium analysis. The cellular pellet was used for growth determination.

Growth determination

The cellular pellet from a culture sample was brought to 10 ml with a solution of crystal violet (0·1 %) and citric acid (0·1 M). After 2 h at 37 °C the stained nuclei were counted on a haemocytometer (Sanford *et al.* 1951).

Amino-acid analysis

Each medium sample was prepared for amino-acid analysis by a single column clean-up procedure, modified from a previously established method (Butler, Darbre & Arnstein, 1975).

Norleucine (0·5 mM) was used as an internal standard. The protein-free supernatant from a culture sample was diluted with 0·1 M-HCl and passed through a Dowex 50W (H^+ form) cation-exchange column (8 cm × 1·1 cm). The resin was washed with 50 ml distilled water adjusted to pH 3. The amino acids were then eluted with 30 ml NH_4Cl (30 ml). A 3 ml sample of this eluate was dried by rotary evaporation and analysed on a D-500 amino-acid analyser (Dionex Corp., Sunnyvale, CA).

Ammonia analysis

The ammonia content of samples taken from cultures was measured by an Orion gas-sensing electrode (model 95-10, Orion Research Inc., Cambridge, MA) connected to a digital meter (model 125, Corning, NY). The electrode was calibrated with standard ammonium chloride solutions, which gave a 56 mV response for a 10-fold concentration change. Free ammonia was released by making solutions 0·1 M in sodium hydroxide immediately before measurement.

RESULTS

Cell growth

Fig. 2 shows the cell growth obtained in a 500 ml culture that was perfused with fresh medium (DMEM + 10 % horse serum) at a rate of 1 ml/min 6 h after inoculation. This represented three volume changes per day. Cell growth showed a biphasic pattern, with a doubling time of 24 h in the first 2 days of growth, while in the next 4 days the doubling time increased to 65 h. The final cell concentration reached 9×10^6 cells/ml in a total of 6 days growth, which involved 3·3 doublings of the original cell inoculum.

Given the available surface area of the microcarriers and the dimensions of the MDCK cells, a monolayer culture should be confluent at $4{\cdot}2 \times 10^6$ cells/ml at a microcarrier concentration of 7·5 mg/ml (Butler & Thilly, 1982). Therefore, at cell concentrations above $4{\cdot}2 \times 10^6$ multilayering would be expected and was in fact observed microscopically. Thus the maximum cell yield obtained was greater than twice the monolayer equivalent based on our calculations. An unperfused culture run simultaneously is shown for comparison (Fig. 2). This culture reached a maximum cell yield of 2×10^6 cells/ml in 3 days at a doubling time of 58 h. The range of yields for MDCK cells grown in unperfused batch cultures has been between 2 and $3{\cdot}5(\times 10^6)$ cells/ml ($n > 10$) in our experience. Initial doubling times have generally

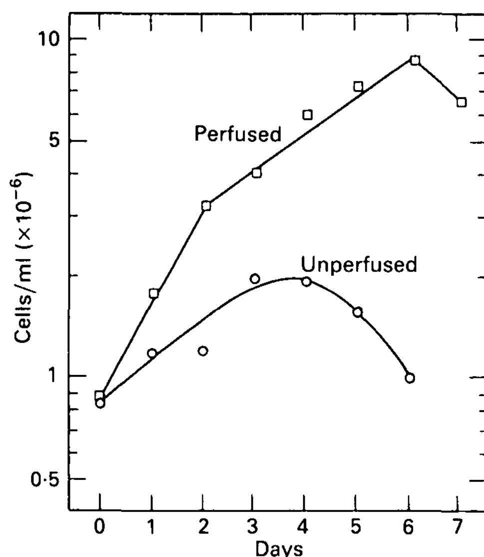

Fig. 2. Growth of cells in a perfused culture compared with an unperfused culture. The MDCK cultures contained 7·5 mg/ml microcarriers in DMEM + 10 % horse serum and were maintained at 37 °C under a 10 % CO_2 overlay. (□) Perfused culture (500 ml) in a 2 l spinner flask. Medium perfusion was commenced 6 h after cell inoculation and maintained at a constant rate of 1 ml/min ($n = 2$). (○) Unperfused culture (100 ml) in a 250 ml spinner flask ($n = 2$).

been close to 24 h, but marked variations as shown in Fig. 2 are occasionally observed.

Culture perfusion with diluted medium

Three perfused cultures were run simultaneously. In all cases the cultures were 200 ml in 500 ml spinner bottles. The initial cell inoculation was performed in undiluted medium (DMEM + 10 % horse serum) and the cultures were incubated with agitation but without perfusion for 6 h to allow attachment of cells upon the microcarriers.

The medium perfusion was run at a rate of 0·4 ml/min, starting 6 h after cell inoculation, at various dilutions of the serum-supplemented medium. Initially PBS was used for dilution, but this failed to maintain a constant pH in the cultures perfused with the 25 % strength medium. Subsequently, a mixture containing the inorganic salts present in DMEM was prepared and used for dilutions. This contained the bicarbonate concentration necessary for maintaining a constant pH of 7·2–7·4 in our cultures under an overlay of 10 % CO_2 in air.

Fig. 3 shows the growth curves obtained from these experiments. Cell densities approaching 4×10^6 cells/ml were found in cultures perfused with undiluted medium and 50 % strength medium. In all cases the cell yields were lower than those obtained in 500 ml cultures as described above. However, significantly higher yields were obtained compared to unperfused cultures (Butler & Thilly, 1982). No significant difference was found in the cell yields obtained in cultures perfused with undiluted

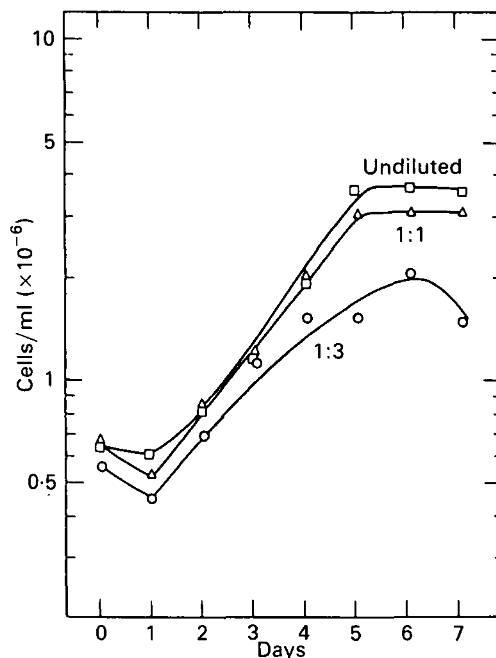

Fig. 3. Growth of cells in perfused cultures at varying concentrations of medium. Cultures (200 ml) in 500 ml spinner bottles were perfused with medium 6 h after cell inoculation. The perfusion rate was 3 vol./day. The cultures contained 7·5 mg/ml microcarriers and were maintained at 37 °C under a 10 % CO_2 overlay. The perfusing medium was: (□) undiluted DMEM + 10 % horse serum ($n = 3$); (△) DMEM + 10 % horse serum diluted 1:1 (v/v) with PBS ($n = 2$) or DMEM salts ($n = 1$); (○) DMEM + 10 % horse serum diluted 1:3 (v/v) with DMEM salts ($n = 1$).

medium compared with the 50 % strength medium. This suggests that the cessation of cell growth at these cell densities is not due to a limitation in medium-supplied nutrients. Either oxygen deprivation or the accumulation of a growth inhibitor is suggested to be limiting growth beyond this point.

Amino-acid utilization

The amino-acid content of the medium was analysed in samples taken from 500 ml perfused cultures at daily intervals. Fig. 4 compares the cellular growth curve with the change in concentration of the 15 amino acids measured.

Three amino acids, alanine, glycine and glutamic acid, showed a net increase in concentration in the medium. Alanine and glycine concentrations also increased in unperfused cultures (Butler & Thilly, 1982). The behaviour of glutamic acid concentrations is noteworthy in that an initial uptake from the medium was followed by rapid production after 2 days culture. This may be related to the rapid consumption of glutamine, which continued over 6 days.

Most of the amino acids showed an initial decrease in concentration followed by stabilization at a level between 60 and 70 % of the original medium concentration. The two notable exceptions were methionine, which decreased to a concentration

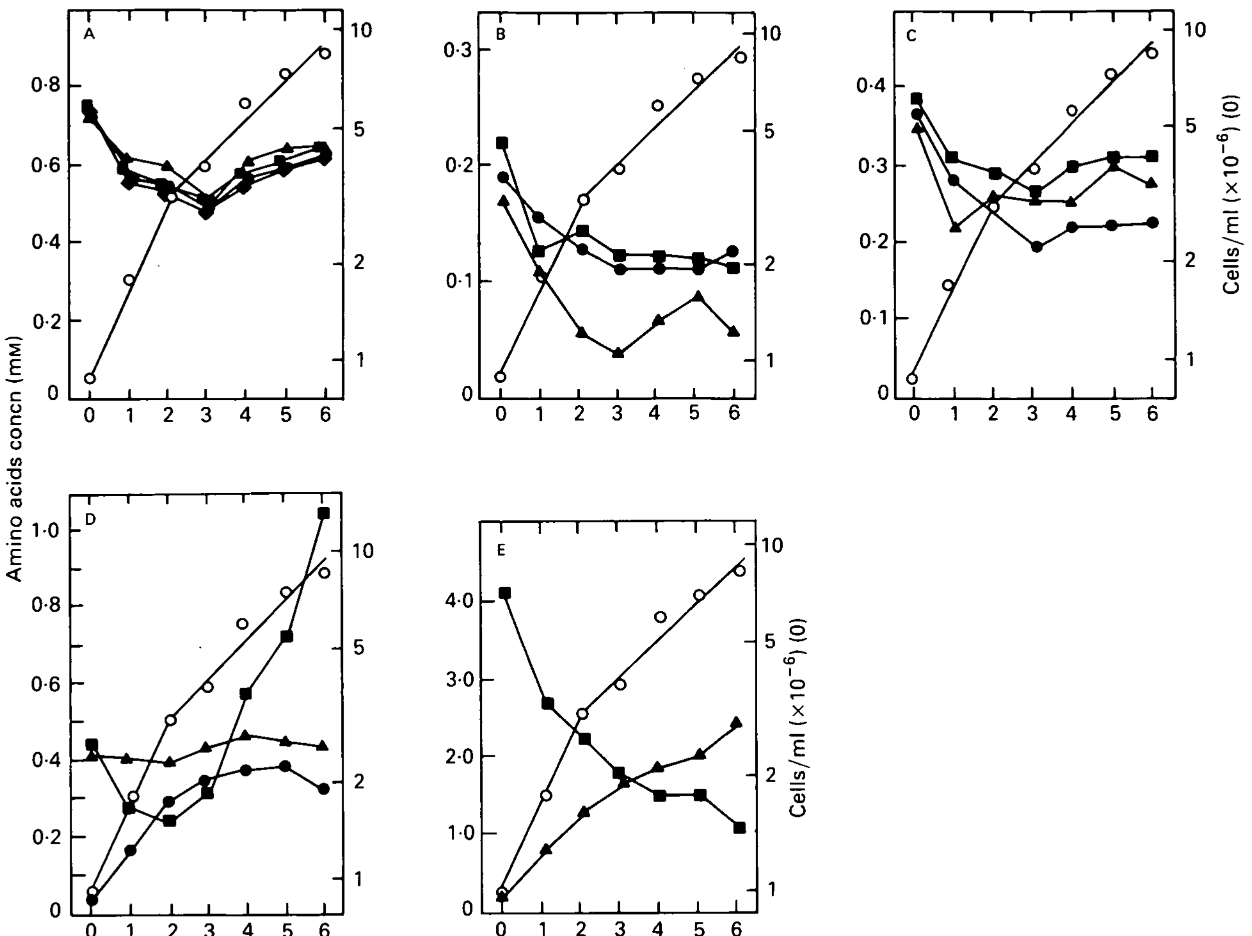

Fig. 4. Concentration of amino acids in the medium of perfused microcarrier cultures of MDCK cells. Amino acid concentrations were measured at daily intervals from samples of a 500 ml perfused culture. Medium perfusion was commenced 6 h after cell inoculation and maintained at a constant rate of 1 ml/min. A. (●) Isoleucine; (◆) leucine; (■) valine; (▲) lysine. B. (●) Histidine; (■) cytidine; (▲) methionine. C. (■) Phenylalanine; (▲) tyrosine; (●) arginine. D. (■) Glutamic acid; (▲) glycine; (●) alanine. E. (▲) Ammonia; (■) glutamine. The corresponding cell growth curve (○) is shown in each case ($n = 2$).

Table 1. *Growth yields of MDCK cells in perfusion culture relative to 15 amino acids and ammonia*

	Perfused		Unperfused‡
	Phase 1*	Phase 2†	
Ala	−3·8	−2·0	−10·1
Arg	5·5	3·4	7·3
Cys	8·8	4·8	35·8
Glu	3·3	−1·7	–
Gln	0·4	0·2	0·8
Gly	22·0	−42·6	−41·4
His	13·2	8·6	40·7
Ile	3·9	3·7	3·9
Leu	3·3	3·1	3·6
Lys	5·5	4·7	11·4
Met	5·7	3·8	20·9
Phe	6·9	5·8	18·5
Tyr	5·1	3·5	8·4
Val	3·3	2·9	4·3
NH_4§	−0·5	−2·5	−2·0

Growth yeild is defined as the number of cells produced per pmole of each amino acid consumed in the perfusion cultures (500 ml).

The quantity of amino acid taken up by the cells during each phase of growth in perfusion was calculated from the following equations:

$$\text{Total content of amino acid supplied to cells} = (Ci)V + kT(Co), \quad (1)$$

where Ci is initial concentration of amino acid in culture (mmol/ml), k the rate of perfusion, T the time of perfusion, and Co is the concentration of amino acid in the perfusion medium (mmol/mol).

$$\text{Final content of amino acid not used by cells} = (Cf)V + kT\int \frac{dC}{dt} \cdot dt, \quad (2)$$

where Cf is the final concentration of amino acid in culture (mmol/ml) and $\int \frac{dC}{dt} \cdot dt$ is the integrated concentration change of the culture over the time period of perfusion.

If a linear decrease in concentration is assumed, then:

$$\int \frac{dC}{dt} \cdot dt = (Ci + Cf)/2.$$

The amino acid uptake by the cells was calculated from the difference between the calculated quantities in equations (1) and (2). For phase 1 of growth only: $Ci = Co$, and the equation can be simplified.

* Phase 1: days 1–2: pre-confluence;

† Phase 2: days 3–7: post-confluent multilayering;

‡ Calculated from Butler & Thilly (1982).

§ Calculated from Imamura *et al.* (1982).

level of 15 % of the original in 3 days, and glutamine, which showed a continuous decrease in concentration to 25 % of its original level in 6 days.

In Table 1 the growth yields of the cells in perfusion culture relative to each of the amino acids analysed that have been calculated are presented. These values offer an index of the efficiency of utilization of the amino acids in terms of cell growth. High positive values indicate favourable cellular productivity in relation to amino-acid consumption. Negative values indicate net production by the cells.

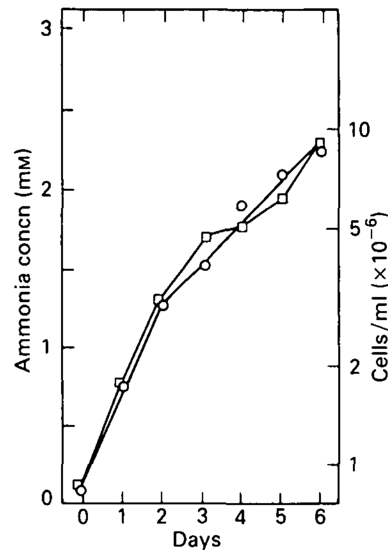

Fig. 5. Concentration of ammonia in the medium of perfused microcarrier cultures of MDCK cells (□) was measured at daily intervals from samples of a 500 ml perfused culture. Medium perfusion was commenced 6 h after cell inoculation and maintained at a constant rate of 1 ml/min. The corresponding cell growth curve is shown (○) ($n = 2$).

The growth yields were calculated for the two phases of cell growth shown in Fig. 2: pre-confluent growth on the microcarriers, and post-confluent multilayering. The lower values observed, post-confluency in most cases, indicate less favourable utilization of amino acids during this stage of growth. In the cases of glutamic acid and glycine a net consumption of amino acids changed into net production. Comparison with values previously determined for unperfused cultures of MDCK cells indicates a similar pattern of utilization (Butler & Thilly, 1982). Glutamine, leucine, isoleucine and valine are among the most highly utilized amino acids under all conditions.

Ammonia production

The ammonia content of samples from the 500 ml cultures was measured with an ion-specific electrode. The concentration of ammonia was found to increase to a value of 2·3 mM in 6 days of cell growth (Fig. 5). This ammonia is thought to arise mainly by cellular reactions, although a proportion may be produced by spontaneous decomposition of glutamine (Tritsch & Moore, 1962). The concentration of ammonia is close to the maximum value previously measured in unperfused cultures of MDCK cells (Imamura *et al.* 1982). Clearly, the rate of perfusion was not sufficiently high to prevent accumulation of ammonia in our cultures. Values for growth yields of cells relative to ammonia production are given in Table 1. Similar values were found in phase 2 of the perfusion culture and the unperfused culture.

DISCUSSION

The potential of medium perfusion in cell culturing was realized by Kruse *et al.* (1963, 1965, 1967, 1969, 1970) who observed multilayered growth of anchorage-dependent cells in T-flasks. The combination of these ideas with developments that have occurred in the growth of mammalian cells on microcarriers (van Wezel, 1967, 1973; Levine *et al.* 1977, 1979; Thilly & Levine, 1979) offers a system of cell production in which yields are considerably higher than have previously been realized. Our final cell densities, which approach 10^7 cells/ml, have to our knowledge never been previously found for microcarrier cultures of mammalian cells. However, Himmelfarb, Thayer & Martin (1969) have grown suspension cultures of murine leukaemia cells to densities approaching 10^8 cells/ml. They used a perfusion system in which cells were separated from medium by using a spin filter device. A spin filter could be employed in a microcarrier system, but we have found the use of the separator column to be both effective and simple. The sedimentation rate of the microcarriers plus cells is sufficient to allow their complete separation from the outflow medium in the separator column.

Multiphasic patterns of cell growth in perfusion cultures were previously observed for a variety of cell types by Kruse *et al.* (1965, 1967, 1969, 1970). The MDCK cells showed a biphasic pattern of growth in which the rate of growth was reduced after a monolayer culture was formed. The cells continued growth to a > twofold monolayer equivalent. Monolayer equivalents greater than unity were observed by Kruse *et al.* (1965, 1967, 1969, 1970) for a number of cell lines in T-flasks.

In unperfused cultures of MDCK cells a plateau of cell density occurs at about 2 to $3(\times 10^6)$ cells/ml (Butler & Thilly, 1982). This cell density is exceeded in perfused cultures and could be due to the supply of nutrients, which becomes limiting, or to the removal of inhibitors that may accumulate. To assess these two possibilities we perfused cultures with medium containing decreased concentrations of carbohydrate source, amino acids, vitamins and serum. The constant rate of perfusion, however, ensured the same rate of removal of cell products. In these experiments we found that exponential cell growth continued to a maximum of 4×10^6 cells/ml in both full-strength and 50% diluted medium. This result suggests that at this level of cell density nutrient limitation is not the critical factor in stopping cell growth.

We were concerned in these experiments with diluted medium because, although we achieved cell yields considerably higher than those of unperfused cultures, the yields were lower than the 9×10^6 cells/ml we had previously achieved. The major difference between these experiments may lie in the culture volume and vessels used. The liquid surface-to-volume ratio of the 500 ml cultures (in which the high densities were observed) was greater than the 200 ml cultures by a factor of nearly 2. Although some oxygen may be supplied to the cells via the perfusing medium, the major source of oxygen would be by diffusion through the surface of the culture. Much work has been devoted to determining acceptable pO_2 values for cells grown in different culture systems (Werrlein & Glines, 1974; Richter, Sanford & Evans, 1972), and a mathematical model has been developed for the diffusion of oxygen in monolayer cultures

(McLimans, Blumenson & Tunnah, 1968). In the light of this information the rate of oxygen utilization in perfused microcarrier cultures may be greater than the rate of oxygen diffusion into the medium – a possibility that is currently under investigation.

Analysis of amino-acid utilization shows that in most cases complete consumption from the medium did not occur during perfusion, which indicates that the perfusion rate was sufficient to supply the cells' amino-acid needs. Two notable exceptions were methionine and glutamine, which were reduced to relatively low concentrations compared to their original values of 0·2 mM for methionine and 4 mM for glutamine.

The large decline in glutamine concentration may be explained, to a large degree, by its metabolic role in cultured cells. Glutamine may be important for many anabolic processes in cells, which include functioning as a lipid precursor (Reed, Zielke, Baab & Ozand, 1981), as a precursor for the biosynthesis of aspartic acid, proline and asparagine (Levintow, 1957), and as a critical component for DNA replication (Zetterberg & Engström, 1981). Its major function though, in terms of mass utilization, may be catabolic in nature by providing carbon skeletons for oxidation via the tricarboxylic acid cycle. In support of this view, carbon dioxide was shown to be a major end-product of glutamine metabolism in cultured cells by several researchers (Lavietes, Regan & Demopoulas, 1974; Stoner & Merchant, 1972; Kovacevic & Morris, 1972). Other investigations have also shown the importance of glutamine as an energy source in human diploid fibroblasts (Zielke *et al.* 1978) and HeLa cells (Reitzer, Wice & Kennell, 1979, 1980).

When amino acids, especially glutamine, are catabolized by cells, ammonia is released as a natural byproduct. Catalysis is so rapid that even in our perfusion system ammonia was produced more rapidly than it was removed and accumulated to a maximum concentration of 2·3 mM. The perfusion system is currently being used as an experimental tool to investigate the effect of ammonia on cell growth, and it is hoped that it will allow experimental discrimination between events that are caused by the increase in ammonia concentration and those that are coincident with the increase.

The advantages of a perfusion system for the culture of cells include the maintenance of growth in a constant environment. Such a stable environment would be suitable for pharmacological studies in which the effect of specific chemicals included in the perfusing medium could be observed on cells at high densities.

Further applications could be suggested in large-scale cell production. The perfusion system has potential for high cell productivity, and this could have important consequences in the economics of industrial production of biological compounds. High cell yields per culture vessel reduce handling, which lowers labour costs as well as the risk of contamination. At present, the perfusion system is an excellent experimental tool for studying the causes of inhibition of cell growth, since a more constant environment can be maintained with respect to both nutrients and growth inhibitors.

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