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ARTIFICIAL CAPILLARY PERFUSION CELL CULTURE: METABOLIC STUDIES

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SUMMARY

Glucose, lactic-acid, and oxygen metabolism of BHK and L929 cells on artificial capillary perfusion units have been studied using several different modes of perfusion. After 7 to 10 days, cells planted in the extracapillary compartment of culture units containing 80 to 150 fibers reached populations that used 0.073 ± 0.025 μmol per min glucose and 0.76 ± 0.26 μl per min oxygen and excreted 0.078 ± 0.038 μmol per min lactic acid. From these data it is estimated that these units contain approximately 2×10^7 cells. The metabolic rate of cultures perfused through the capillaries or through the extracapillary compartment was not affected significantly by change in flow rate except at perfusion flow rates ≤0.05 ml per min. The cell population, as measured by metabolic activity, did not increase significantly when the serum content of the medium was ≤1%. No major differences were found in glucose utilization rates of equal numbers of cells on artificial capillaries, on short-term suspension culture, or as monolayers in plastic flasks. Artificial capillary perfusion may provide a simple system for studying metabolism of mammalian cells in culture.

Key words: metabolism; artificial capillaries; perfusion cell culture.

INTRODUCTION

In 1972, Knazek et al. (1) described a perfusion system that used semipermeable hollow fibers in a tube and shell configuration to support cell growth. Since then a number of reports have appeared that describe the use of artificial capillary systems for perfusion cell culture (2 to 15). Fibroblast cells, including diploid (4,5,9,12,13), aneuploid (1-3,7,14), and nonfibroblast cell types (8,15) have been maintained successfully on such units for periods as long as 3 months. Tissue-like densities can be achieved (1), and hormone production per cell can exceed that expected from the same cells maintained in flask cultures (2).

Artificial capillary perfusion cell culture is of potential importance in the development of artificial organs, the harvesting of secreted hormones (2,9), investigations of blood-vessel biochemistry (*Personal communication*, H. P. Chin, Dept. of Medicine, University of Southern California, Los Angeles; and R. G. Mason, Dept. of Pathology, the Memorial Hospital, Pawtucket, Rhode Island), studies of density effects and nutritional requirements of cells in culture, short-term toxicity studies (16), and, possibly, the improvement of growth in vitro of otherwise hard-to-grow cell types.

In order to develop a cell culture toxicity detector, we have been routinely culturing BHK or L929 cells (hamster and mouse fibroblast cell lines, respectively) on artificial capillaries. To estimate planting success, the glucose and oxygen utilization rates and lactic-acid excretion rates have been measured. Several different perfusion modes on seven different culture unit devices have been used: the recirculation perfusion system of Knazek et al. (1); once-through perfusion (at low flow rates) of culture medium through the capillaries; and once-through perfusion through the extracapillary compartments.

MATERIALS AND METHODS

Cell culture. Stocks of BHK/21 Cl 13 cells and L929 cells from the Microbiology Department at Louisiana State University, New Orleans, were grown at 37° C in closed plastic flasks (Falcon) in Dulbecco's minimal essential medium (H 16, GIBCO) supplemented with sodium bicarbonate (0.35 g per l), sodium chloride (2.0 g per l), and bovine serum (5%, KC Biological). Where indicated, chlorotetracycline (100 μg per ml, Sigma), penicillin-G (100 U per ml, Sigma), and streptomycin sulfate (100 μg per ml, Sigma) were added.

The cells were subcultured once a week at split ratios of 1:4 or 1:5 using 0.25% trypsin (Difco) in phosphate-buffered saline.

For artificial capillary perfusion cultures, 1 to 2 × 10^7 cells were planted in the extracapillary space of units described in Table 1. The units were placed in sterilized perfusion circuits (Fig. 1*a*) and perfused with medium containing antibiotics and 20 mM HEPES, pH 7.3, at flow rates ranging from 2 to 10 ml per min using a Brinkman 6-channel peristaltic pump. The entire planting operation took less than 45 min. Cultures were maintained for the 1st week with medium containing 5% bovine serum (50 to 100 ml per reservoir). During this time the reservoir medium was replaced twice. After the 1st week, medium with only 1% bovine serum was used to perfuse the cultures. Contamination checks were made by culturing aliquots of the reservoir and the extracapillary medium in brain heart infusion broth and thioglycollate medium (Difco). Aliquots were taken periodically from the reservoir medium for measurement of lactic acid (Sigma test kit 826UV) and glucose (Sigma test kit 510) content.

The oxygen content of the medium entering and leaving the culture unit was measured amperometrically by an Instrumentation Laboratories Model 113 blood/gas analyzer. Before use, the instrument was calibrated with nitrogen and 11.82% oxygen gases. Drift from calibration over 12 hr was less than 7 mmHg. The culture unit was connected directly to the microelectrode chamber of the oxygen meter.

Cell number was determined by counting in a hemacytometer after removal of most of the cells from the unit by incubation for 30 min at 37° C with 0.25% trypsin in phosphate buffered saline. The trypan blue exclusion method was used to determine the numbers of viable and nonviable cells. Additional treatments of the culture units with trypsin did not yield significant further recovery of cells.

Capillary units. The artificial capillary units (Table 1) were purchased from Amicon (Vitafiber 3X50 or 3S100) or were manufactured at Gulf South Research Institute (GSRI). The GSRI units were constructed from polycarbonate tubes with sidearms attached to the shell. The fibers (cellulose acetate) were sealed into the shells by the method described by Knazek et al. (1) using RTV-11 silicone rubber (General Electric) or Vorite-689 287-70G, a urethane polymer (NL Industries). The units were washed extensively with running tap water by flow through the fibers and by ultrafiltration to ensure removal of toxic substances that were used in the fiber spinning. The Amicon-3S100 units were sterilized by autoclaving; the other units were sterilized by immersion in 2.5% formaldehyde for 1 day followed by 70% ethanol for at least 1 week.

Perfusion mode. Perfusion of the artificial capillaries was by the recirculating system shown

TABLE 1

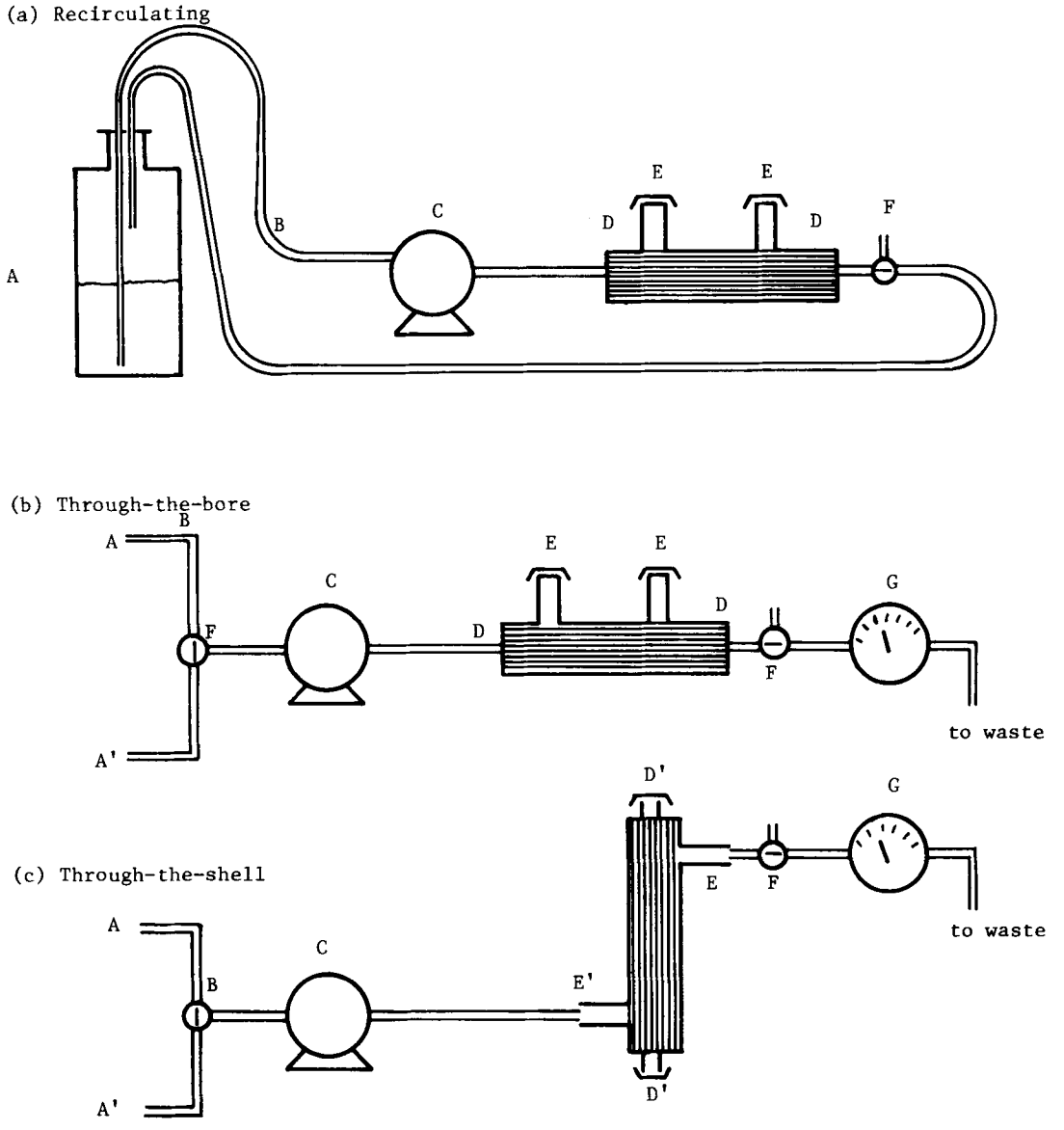
PROPERTIES OF CULTURE UNITS USED FOR ARTIFICIAL CAPILLARY PERFUSION

	Amicon-3S100	Amicon-3X50	GSRI-75	GSRI-150	GSRI-300	GSRI-500	GSRI-80
Capillary type	Polysulfone	Acrylic copolymer	Cellulose acetate	Cellulose acetate	Cellulose acetate	Cellulose acetate	Cellulose acetate
Potting	Epoxy	Epoxy	Silastic	Silastic	Urethane	Urethane	Urethane
Number of fibers	150	150	75	150	300	500	80
I.D. (μm)	200	200	275	109	250	250	250
O.D. (μm)	350	350	475	200	350	350	350
Total area, fiber surface (cm^2)	94	94	48	57	307	511	52
Intracapillary volume (cm^3)	0.27	0.27	0.269	0.09	0.86	1.45	0.23
Extracapillary volume (cm^3)	2.6	2.6	0.8	0.6	14	12	2.1
Molecular weight permeability	100,000	50,000	See below[a]	See below[a]	See below[a]	See below[a]	See below[a]
Water permeability $L_p\left(\frac{cm}{atm\ sec}\right)^b$ (× 10^5)	240	80	747	970	400	400	400

[a] Permeable to bovine serum albumin.

[b] L_p was measured on the GSRI units before planting with cells. For the units with fibers potted in Silastic, the fibers were not glycerinized or dried before potting. On Amicon units the L_p was measured on units that had been used to grow cells and subsequently were trypsinized and washed. Static L_p was measured at 200 mmHg pressure. For the GSRI-80, -300, -500 units, fibers from the same spinning were used.

in Fig. 1*a*. After 10 to 14 days from the date of planting, in certain experiments, units were incorporated into once-through perfusion systems. In these, which perfuse the culture medium through the bore of the fibers (Fig. 1*b*) or through the extracapillary space (Fig. 1c; through-the-shell), slow flow rates (0.05 to 1.24 ml per min) were used instead of the faster rates (>2 ml per min) used for recirculating flow. The second reservoir indicated in Fig. 1*b,c* could be used to introduce medium containing a toxic substance. Flow rate was measured by timing the accumulation of



A — Revervoir for tissue culture medium
A′— Reservoir for tissue culture medium plus toxin
B — Silastic tubing
C — Peristaltic pump
D — Male Luer connectors
D′— Male Luer to closed end
E — Female Luer to closed end
E′— Female Luer connectors
F — Luer-3-way stopcock
G — Oxygen meter

FIG. 1. Perfusion schemes for artificial capillary cell culture.

TABLE 2

AVERAGE GLUCOSE CONSUMPTION AND LACTATE EXCRETION BY BHK AND L929 CELLS ON ARTIFICIAL CAPILLARIES[a]

Cells	Glucose Consumption	Lactate Excretion	No. of Experiments
	μmol/min	*μmol/min*	
BHK	0.072 ± 0.020	0.086 ± 0.034	9
L929	0.075 ± 0.037	0.065 ± 0.047	5

[a] Metabolic activity was determined 1 to 2 weeks after planting.

waste medium. The rate was adjusted to allow measurement of a metabolic parameter such as oxygen uptake or lactic-acid accumulation. In the once-through perfusion modes, either Tygon or silicone rubber tubing was used to connect the reservoir to the unit.

RESULTS AND DISCUSSION

Cell growth on artificial capillaries. The average glucose-utilization and lactic-acid excretion rates for BHK and L929 cells grown on artificial capillary units obtained from Amicon (3S100) or GSRI (75 or 150) are given in Table 2. These units were cultured according to the protocol given in Materials and Methods in which, after 1 week, medium with 1% bovine serum was used to feed the cells. Following this change, no significant increase in glucose utilization was observed, indicating that cell population was not increasing. With an estimated average glucose utilization rate of 5 μmol per 10^6 cells per day (17), the cell population was 21 ± 8 × 10^6 cells within 2 weeks after initial planting with 1 to 2 × 10^7 cells. This estimate is based on the average glucose-utilization rate for a proliferating cell population. Since glucose utilization increases in postconfluent cultures (17) of certain cells, the estimated cell number may be higher than the actual one. The actual viable cell number recovered from the units after trypsinization was 13 ± 8 × 10^6 cells. This decrease in the expected amount of viable cells may be due to the prolonged trypsinization resulting in loss in cell viability or the inaccessibility to trypsin (or entrapment) of living cells on the fibers. Many of the cells in the initial population settle on the outer walls of the shell and do not survive, presumably due to inadequate supply of nutrients from the perfusion medium. Our observation that after several days many cells were floating in the units and took up vital dye is in agreement with the observations of other workers (1,7) of an initial loss of cell viability following planting.

With the use of rather heavy cell inocula (1 to 2 × 10^7 cells per unit), almost all of the culture units were able to support a cell population of at least 2 × 10^7 cells (as evidenced by glucose utilization). No significant advantage was found, in terms of cell growth, with the cellulose acetate fiber units compared to the Amicon polysulfone or acrylic copolymer fibers. The larger shell volume of the Amicon units, as compared to the GSRI—75 or -150 units, however, allowed more cells in the initial inoculum to settle on the shell walls rather than to adhere to the fibers. This, however, did not appear to delay the initial growth as determined by comparing the glucose utilization in Amicon and GSRI units at early times after planting (*results not shown*).

In the studies of glucose and lactic-acid metabolism of cells on 14 units averaged in Table 2, perfusion was with a recirculating system (Fig. 1*a*) with flow rates ranging from 2 to 8.5 ml per min. With the same inoculum, more than twice the population of L929 cells (0.5 × 10^6 cells per cm^2) was achieved on an Amicon-3S100 unit by using a flow rate of 8.5 ml per min as compared to 2 ml per min (0.2 × 10^6 cells per cm^2) for recirculating perfusion of the cells. With even higher flow rates, 100 ml per min (*Personal communication*, J. Dietrich, Abbott Laboratories), cell densities of 0.8 × 10^6 cells per cm^2 have been found (on a GSRI-500 unit using HeLa cells). The

TABLE 3

EFFECT OF SERUM ON METABOLIC ACTIVITY[a]

Day[b]	5% Serum		1.0% Serum		0.1% Serum	
	Glucose	Lactic Acid	Glucose	Lactic Acid	Glucose	Lactic Acid
	μmol/min	*μmol/min*	*μmol/min*	*μmol/min*	*μmol/min*	*μmol/min*
4	0.081	0.114	0.045	0.064	0.043	0.065
6	0.097	0.146	0.062	0.062	0.040	0.073

[a] L929 cells were cultured identically on Amicon-3S100 units. After 1 week the glucose utilization rate was 0.045 ± 0.012 μmol per min and the lactic-acid excretion rate was 0.068 ± 0.021 μmol per min. At this time the original medium was replaced with medium containing different serum concentrations. Cultures at each serum level were in duplicate and the values are averages.

[b] After change from 10% serum medium to medium of different serum concentrations.

faster flow may improve nutrient supply to the cells on the fibers due to the Starling effect (18) causing increased diffusion of substances between bore and shell fluids.

The serum content of the medium also has been found to affect cell growth on artificial capillaries (Table 3). After change from medium with 5% serum to medium with 1% or 0.1% serum, the glucose utilization and lactic-acid excretion rates stabilize; with prolonged culture using 5% (or 10%) serum content, maximal cell numbers (recovered by trypsinization) of 5×10^7 (GSRI-150) and 6×10^7 (Amicon-3S100) corresponding to glucose utilization rates of 0.130 and 0.165 μmol per min, respectively, were achieved. The cessation of growth of cells in medium containing low serum has been found for cells in other perfusion systems (17) and in flask cultures (19,20). Cell growth was the same when the reservoir contained 100 ml rather than 20 ml of medium. It was anticipated that "conditioning" factors not provided in the medium might be necessary to initiate healthy growing populations of cells. For L929 and BHK cells, such factors either are not required in high concentrations or are of sufficient molecular weight to be prevented from diffusing through the walls of the artificial capillaries and therefore remain at high concentration in the shell medium.

The 170 cm of Silastic tubing used in the perfusion circuit provided adequate although incomplete reequilibration of the deoxygenated medium with atmospheric oxygen. With a cell population that used 1.0 μl oxygen per min, the reservoir pO_2 was 128 mmHg, the unit outflow pO_2 was 116 mmHg, and the pO_2 of the shell volume was 118 mmHg at a perfusion flow rate of 1.24 ml per min. In contrast, the initial medium oxygen content was 142 mmHg.

Once-through perfusion cell culture. Once-through perfusion either through-the-bore of the capillaries (Fig. 1*b*) or through-the-shell (Fig. 1*c*) was performed on cultures that were established by use of a recirculating perfusion system.

The effects of changes in flow rate on glucose utilization and lactic-acid excretion of cultures perfused as in Fig. 1*b* are shown in Fig. 2*a*, and on oxygen consumption in Fig. 2*b*. As flow rate decreases, the glucose utilization and lactate excretion increase (Fig. 2*a*), presumably due to the Pasteur effect (21), since oxygen depletion of the medium occurs (Fig. 2*b*) as the flow rate slows. With a slow flow rate, a portion of the cells would be in anaerobic culture and, therefore, should consume more glucose to satisfy their energy requirements (21). At the flow rates for which oxygen utilization is measured, the utilization rate does not change significantly with flow rate. The effect on cell viability of prolonged culture (days) under partially anaerobic conditions has not been determined; however, a decrease in glucose utilization, possibly due to loss in cell viability, was apparent when cultures were perfused at low flow rates for several hr (Table 4). For the studies in Table 4, a perfusion flow rate of 5 ml per min was used initially. At this flow rate, the glucose utilization and lactate excretion rates for these cultures were 0.130 ± 0.028 and 0.135 ± 0.031 μmol per min, respectively. After perfusion for several hr at 0.05 ml per min, however, the glucose utilization was one-third and the lactic-acid excretion one-half of that found at the faster flow rate (Table 4). This decrease in the glucose and lactic-acid metabolism rate is opposite to the increase caused by the Pasteur effect (Fig. 2*a*), which was measured on cells exposed to slow flow for only 1/2 hr. As can be seen in Table 4, essentially no difference in metabolic activity was found between through-the-shell or through-the-bore perfusion. In recirculating perfusion at fast flow rates, the lactate and glucose metabolic rates

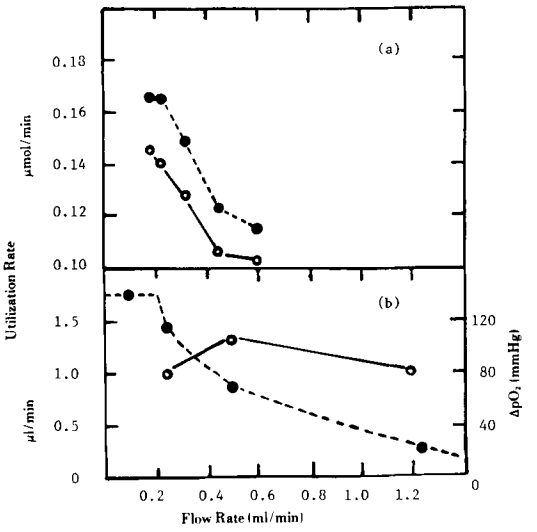


FIG. 2. Effect of flow rate on glucose and oxygen utilization and lactic-acid excretion. *a*, Glucose (*solid line*) and lactic-acid metabolism (*dashed line*). BHK cells on GSRI-150 unit; once-through flow-through-bore perfusion. Cells were planted 2 weeks prior to use in this study. *b*, Oxygen utilization. BHK cells on an Amicon-3S100 unit, flow-through-bore perfusion. Cells were in culture on the unit for 2 weeks. ΔpO_2 (mmHg) (*dashed line*) is the inlet medium oxygen content (141 mmHg) minus the outflow oxygen content. Utilization rate (*solid line*) (μl per min) = flow rate (ml per min) times ΔpO_2 (mmHg) times 0.037 μl per mmHg (at 37° C).

TABLE 4

LACTIC ACID AND GLUCOSE METABOLISM: ONCE-THROUGH PERFUSION[a]

Mode	Glucose	Lactic Acid	No. of Experiments
	μmol/min	*μmol/min*	
Through Bore	0.045 ± 0.011	0.074 ± 0.017	3
Through Shell	0.043 ± 0.016	0.097 ± 0.019	3

[a] BHK cells on a GSRI-75 culture unit perfused at 0.05 ml per min. Cells were established in recirculating perfusion culture at 5 ml per min.

are approximately the same (Table 2); whereas in once-through perfusion at rates sufficiently slow to allow depletion of the medium oxygen by the cells (Table 4), the lactate excretion is close to twice that of the glucose utilization. A possible explanation of this observation is that with adequate aeration a greater amount of the cellular ATP is derived from conversion of glucose to CO_2 and, therefore, a lower net yield of lactate is produced. However, under anaerobic perfusion (perfusion at slow flow), the end product of glucose metabolism is predominantly lactate rather than CO_2.

The oxygen utilization rates for cell cultures on a variety of units perfused either through-the-shell or through-the-bore are given in Table 5. These data are for cells in culture on the units for 1 week. Except for the larger units (GSRI-300 or -500), the oxygen utilization rates were less than 1.0 μl per min for BHK and L929 cells. The Amicon-3X50 and GSRI-80 units averaged lower cell populations than did the Amicon-3S100 or GSRI-150 culture units with fibers of comparable areas. There was no significant difference between the average oxygen utilization of small units perfused through the bore compared to that of units perfused through the shell (Table 5). For the larger GSRI-300 and GSRI-500 units, the average oxygen utilization for through-the-bore perfusion was slightly higher than for through-the-shell perfusion. It is probable that as the number of cells on a unit or the size of a unit increases, the efficiency of medium exchange by through-the-shell perfusion is less than for the through-the-bore perfusion. The fact that no difference was found with small units, which have relatively low populations (20×10^6 cells per unit), implies (a) that the nondiffusible serum factors (globulins, for example) are not limiting cell metabolism in the through-the-bore mode (these would be more available in through-the-shell perfusion); and (b) that the accessibility of cells to the medium is equally adequate for good growth with both methods of perfusion.

TABLE 5

AVERAGE OXYGEN UTILIZATION BY BHK AND L929 CELLS ON ARTIFICIAL CAPILLARIES[a]

Artificial Capillary Unit	Oxygen Uptake Through-Bore	Oxygen Uptake Through-Shell
	μl/min	*μl/min*
3X50	0.49 ± 0.23 (7)[b]	0.59 ± 0.22 (7)
GSRI-80	0.46 ± 0.29 (5)	0.56 ± 0.21 (5)
3S100	0.88 ± 0.41 (9)	0.99 ± 0.11 (5)
GSRI-150	0.71 ± 0.31 (6)	0.92 ± 0.14 (2)
GSRI-300, 500	1.73 ± 0.45 (3)	1.19 ± 0.21 (2)

[a] Cells were established on perfusion circuits as in Fig. 1*a* for 1 week and then transferred to once-through perfusion systems. Utilization rate (U) is determined as the flow rate times the ΔpO_2 (mmHg) times 0.037, the amount of oxygen (μl) per mmHg in medium at 37° C.

[b] Number in parenthesis is number of individual units tested. Each measurement was made within 1 hr after transfer to the once-through perfusion mode.

Assuming the oxygen consumption per 10^6 cells to be 0.047 μl per min (16), the cell population on the small units is estimated to be 17×10^6 cells per unit. Within experimental error this number is in agreement with that estimated from glucose utilization rates, namely 21×10^6 cells per culture unit, and cell number from trypsinization (13×10^6 cells). In separate studies we have found that cultures could be initiated at slow flow rates using through-the-bore perfusion and that cultures could be maintained for as long as 15 days using once-through perfusion. The use of once-through perfusion for studies of toxicity has been reported separately (16). Other potential uses of once-through perfusion for harvesting excretion products, for studies of cell metabolism, and for use of cells as enzyme reactors can be envisioned.

Comparison of cell metabolism on artificial capillaries in culture flasks or in suspension. An experiment was performed to compare the metabolism of cells on artificial capillaries with their metabolism in flasks or in suspension (Table 6). For this study the cells were cultured in units 1 week before study. Perfusion was at 1.24 ml per min. The flask cultures were initiated 24 hr before study; cells were close to confluency. For suspensions, the cells were trypsinized and kept in suspension by slow magnetic stirring. Cell counts were made at the end of each test of a particular culture method. As discussed earlier, the cell counts for culture units may be low due to the incomplete removal of the cells by trypsinization. The rates were calculated from the slopes of the lines through the points, relating amount of utilization (glucose) or excretion (lactic acid) with

TABLE 6

COMPARISON OF METABOLIC ACTIVITY OF CELLS ON CAPILLARY UNITS IN SUSPENSION AND IN FLASKS[a]

Culture Condition	L929 Cells		BHK Cells	
	Glucose Utilization	Lactate Excretion	Glucose Utilization	Lactate Excretion
	μmol/min	*μmol/min*	*μmol/min*	*μmol/min*
GSRI-150	0.078	0.087	0.096	0.106
Amicon-3S100	0.064	0.075	0.062	0.089
Flask[b]	0.079	0.148	0.066	0.130
Suspension[c]	0.074	0.111	0.074	0.180

[a] Rates are given as μmol per min per 2×10^7 cells. Cells on artificial capillaries were maintained at a flow rate of 1.24 ml per min by a perfusion circuit (reservoir volume, 25 ml). The cells were established 1 week before use. The experiment was performed in duplicate and the values given are averages. The average variation was ± 0.020 μmol per min for glucose and ± 0.032 μmol per min for lactic-acid measurements.

[b] Falcon plastic flasks (75-cm^2).

[c] In Erlenmeyer flasks. For all cultures, the medium contained 20 mM HEPES, pH 7.3.

time. The final medium glucose concentration after each study was greater than 3 mM. For each test, aliquots were taken at five time points. The results indicate that for these cell populations (2×10^7 cells per unit) and a relatively fast flow rate, the glucose metabolism rate of cells on artificial capillaries is not significantly different from that of cells in flask or suspension culture. The lactate excretion rate is almost twice the rate of glucose uptake in the flask and suspension cultures but is approximately equal to the glucose rate for the capillary cultures. Since nonshaking monolayer cultures of nearly confluent cells are virtually anaerobic at the cell surface (22), most of the glucose, in such cultures, is converted to lactate by glycolysis. The lactic acid/glucose ratio may be high for the cells in suspension culture if the cells receive an inadequate supply of oxygen due to slow reequilibration of the depleted medium with the air. As suggested above, in the capillary perfusion system of Fig. 1*a*, the close proximity of the oxygenated medium stream with all the cells prevents oxygen starvation and allows relatively more of the energy requirements of the cell to be derived from respiration, thereby lowering the observed lactic acid/glucose ratio.

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