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A DEVICE FOR MAINTAINING VIABLE CELLS AT HIGH DENSITIES
FOR NMR STUDIES

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In recent years there has been increased interest in the use of NMR to study living cells (1,2). Because the technique is relatively insensitive, cells must be studied at high densities. Thus investigators have developed methods of maintaining dense pellets of healthy, stable cells in a magnet for long periods of time. In early studies of aerobic cells oxygen was supplied from a bubbling apparatus, with no attempt to supply nutrients or remove waste products on a continuous basis (3). Recently methods have been developed which allow the study of anchorage dependent cells which are on solid supports and are continuously perfused with oxygenated media. Ugurbil et al (4) allowed cells to attach to Cytodex beads (Pharmacia) and perfused the cells with medium while NMR spectra were acquired. Spectra have also been obtained from cells grown in commercially available Vitafiber Units (Amicon) (5). A disadvantage of these approaches is that the solid supports occupy most of the radiofrequency coil volume and as a result there is a significant loss in sensitivity. In addition the devices required are not compatible with conventional NMR probes, which are accessible only from the top. Neither technique has been used with cells which are not anchorage dependent.

We have developed a system which maintains cell suspensions in a steady state at densities necessary for NMR experiments and is directly compatible with conventional probes. Using hollow fiber dialysis tubing to perfuse the cell pellet with oxygenated medium, we have obtained ^{31}P spectra of secondary chicken embryo fibroblasts and a free living protozoan, *Tetrahymena thermophila*.

Fig. 1 is a schematic illustration of our apparatus. A bundle

of cellulose acetate fibers, occupying approximately one sixth of the sample volume is sealed into the ends of polyethylene tubing using water resistant epoxy (2 ton epoxy, Devco). The fibers are then inserted into an NMR tube whose top has been modified so that a pressure tight seal can be made around the polyethelene tubing. Nutrient rich medium is pumped through the fibers at a rate of 5 - 10 mls/minute by a peristaltic pump. The fibers are semipermeable, allowing molecules with a molecular weight of less than one thousand Daltons to pass in and out of the cell pellet.

The peristaltic pump generates a pressure difference across the walls of the fibers, causing the level of fluid in the sample tube to change, even though there are no breaks in the fibers or in the epoxy. If the peristaltic pump is used to force fluid into the fibers, thus producing a positive pressure inside the fibers, the fluid level inside the sample tube increases. Alternatively if the pump is used to produce a lower pressure inside the fibers, the fluid level in the sample tube decreases. Since changes in cell density will affect NMR results, we devised several methods for keeping the fluid level constant. The sample tube is hermetically sealed, so that as medium is pumped through the fibers the fluid level increases until a limiting pressure is reached. The limiting pressure will depend on the flow rate, and the number and type of fibers used; our apparatus operates at a pressure of 4 psi. The system reaches equilibrium at this point, and there is no further increase in fluid level or in pressure. To minimize the initial increase in fluid level which occurs before the pressure equilibrates, a tightly fitting nylon plug occupies most of the empty space inside the tube.

The equilibrium pressure is reduced considerably when the peristaltic pump is used to equalize the input and output pressures, i.e. to push on the supply tube and pull on the return tube.

When fibers with relatively large pores are used (Celanese Celgard), and the pump maintains a low pressure inside the fibers, there is a substantial flow of liquid from the cell suspension into the fibers. Because the sample tube is airtight there is a compensating flow of liquid down the vent line and into the sample tube maintaining a constant cell density. This arrangement has the advantage of providing a net flow of medium through the pellet.

Another approach is to imbed the cells in an agar/collagen gel which holds the cells in place in the sample tube. The fluid level is then allowed to increase without affecting cell density while excess medium is removed through the vent line. In this mode of operation a pressure seal is not required so that unmodified NMR tubes may be used.

The data in Fig. 2 demonstrate the ability of the fibers to transport glucose, lactate, oxygen and protons. The rate of glucose and lactate transport can be as high as 20 mgs/hour. Oxygen reaches equilibrium much more rapidly than do the other solutes, presumably because it is carried by water so that the rate of oxygen transport is the same as the rate of flow of water across the fiber membranes. The equilibration of pH is slow compared to that of oxygen transport (Fig. 2D), indicating that hydronium ions are transported more slowly than water. In general the rate of equilibration of pH may be strongly influenced by the permeabilities of the protonated forms of weak bases (i.e. phosphate and lactate) which are present in solution. The rates

of transport are strongly dependent on the size of the concentration gradients across the hollow fiber membranes. We maintain large gradients by changing the perfusate at frequent intervals, and bubbling oxygen into the reservoir. When the glucose concentration in the perfusate is kept at its normal level (1 mg/ml) the rates of transport are in excess of what is required to satisfy the needs of our cells; confluent CEFs on culture dishes use 15 ugms of glucose / ten to the sixth cells/hour, and they secrete lactate at a similar rate. When the level of glucose in the perfusate is very high it may be necessary to use more fibers in order to remove the lactate which the cells produce. Fig. 3 shows the response of the cells as glucose is delivered through the fibers. When the perfusate contains no glucose the cells do not produce ATP; when glucose is added to the perfusate the ATP level increases within 20 minutes. Once the maximum ATP level is achieved, it can be maintained for up to 48 hours.

Tetrahymena thermophila consume oxygen at high rates (4.2 umoles/min/ gm wet weight) and thus provide us with a good test of the fibers' ability to deliver oxygen. The inset to Figure 4 shows the oxygen tension of a suspension of *Tetrahymena* at densities necessary for good sensitivity in an NMR experiment. As an oxygenated solution is pumped through the fibers, the Oxygen level rises from zero and achieves a steady state within minutes. The spectra in Fig.4 show that ATP levels detected by NMR rise as oxygen is delivered to the cells from the dialysis fibers, reaching levels equal to those obtained by bubbling oxygen directly into the cell pellet.

In conclusion, we are able to maintain cells at high densities for long periods of time in a superconducting magnet. The

levels of oxygen and other nutrients are kept at adequate levels and waste products are removed continuously. As a result the cells can be maintained in homeostasis or modulated during lengthy NMR experiments. This method is particularly useful for cells whose growth is not anchorage dependent (i.e. protozoa, virally transformed cells, lymphocytes, etc.) although a combination of the fiber system with cells attached to beads is a possibility.

We are using the technique described here to study regulation of glycolysis in normal and virally transformed chicken embryo fibroblasts, and rates of ATP turnover in *Tetrahymena thermophila*.

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FIGURE CAPTIONS

Figure 1

The NMR sample holder is a glass tube, 13.5 mm I.D., epoxied to a nylon top. Three polyethylene tubes enter the sample holder; one carries medium flowing into the fibers, one carries medium leaving the fibers, and one is a vent tube. An O ring is placed around each tube, between two pressure plates. A threaded top screws down on the pressure plates, compressing the O rings and creating a seal at the point at which the polyethylene tubes enter the sample holder. At the same time, the bottom pressure plate compresses a fourth O ring creating a seal around the circumference of the sample holder. Dialysis fibers, which are epoxied into the polyethylene tubes, are distributed evenly throughout the two ml sample volume.

Figure 2

Transport properties of dialysis fibers: a) Glucose transport; fluid is pumped from a 75 ml reservoir of Medium 199 with 5mM glucose through fibers in a test tube containing 10 mls of Med 199. Graph shows concentration of glucose in the test tube. b) Lactate transport; fluid is pumped from a 75 ml reservoir of Medium 199 through fibers immersed in 25 mM lactic acid. Graph shows concentration of lactic acid in reservoir. c) Oxygen transport; fluid from an air saturated reservoir is pumped through fibers immersed in a test tube containing 5 mls of O₂ depleted water. Graph shows O₂ tension in the water. d) Equilibration of pH; Phosphate buffer at pH 6.8 is pumped through fibers in a test tube with 10 mls phosphate buffer at pH 4.9. Graph shows pH in test tube.

Figure 3

Effect of glucose on metabolism of CEFs. Cells were grown as previously described(6), removed from culture dishes using trypsin and pelleted at 200 xg. ^{31}P spectra were taken at 109 MHz using 45 degree pulses and a recycle time of 200 Msec. Each spectrum is the average of 3000 free induction decays and an exponential filter of 20 Hz was applied. a) CEFs perfused with glucose free Medium 199. b) 5 mgs/ml glucose added to the perfusate. Peak assignments are: 1) glucose-6-phosphate and fructose- 6-phosphate 2) inorganic phosphate 3) gamma NTP 4) alpha NTP 5) beta NTP.

Figure 4

Effect of perfusing Tetrahymena with Tris buffer (pH 7.2) equilibrated with 100% oxygen. Tetrahymena thermophila BIV 1868 were grown to mid-log phase at 30deg. then harvested by centrifugation at 200 xg at 4deg. The resulting pellet was transferred into the flow cell for NMR or a duplicate chamber containing both fibers and a Clarke type oxygen electrode (YSI). ^{31}P spectra were obtained at 109MHz at 25deg using a 45deg pulse and a 206Msec delay between pulses. a) Spectrum obtained before buffer is passed through fibers. b) Spectrum obtained 10 min. after the pump has been switched on. Peaks shown are 1) Methylene Diphosphonic Acid (pH 9), contained in an external capillary 2) inorganic phosphate 3) gamma NTP 4)alpha NTP, NAD(H) 5)beta NTP.

The inset shows the effect perfusion has on the oxygen tension of the cell pellet.

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Figure 1

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Figure 2

XBL 8210-4192

Figure 3

a) Pump Off

b) Pump On

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Figure 4

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