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Multinuclear NMR Studies of an Actively Dividing Artificial Tumor

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Summary

Growth of the A549 cell line in a perfusion system suitable for use in a magnetic resonance study has been characterized and shown to be stable physiologically and hence appropriate for serial observations. Several methods of monitoring cell growth were compared to assess the behavior of the cells in this system. Comparison between NMR metabolite data and cell growth *via* cell counting showed that ^{31}P NMR signals accurately reported cell doubling time. In contrast to most NMR cell culture systems, viable cells can be recovered from the perfusion system after the NMR measurements for further biochemical studies. These data further suggest that this system will be useful for studying the physiology and biochemistry of exponentially growing cells for at least two days in NMR tube culture.

Key words
A549 • Phophorous • NMR • Cell culture

Introduction

Magnetic resonance spectroscopy is uniquely suited to address problems in metabolism and biology *in vivo*. In particular, resonances from NMR detectable nuclei are being studied to measure processes involving biologically important molecules. To simplify interpretation of spectral changes associated with complex biochemical systems, cellular model systems are being developed. These systems simulate *in vitro* isolated organs with healthy, growing cells continuously provided with nutrients and oxygen. Several cell perfusion systems have been described in the literature as artificial organ systems to study tumors (Ugurbil *et al*. 1981, Gonzalez-Mendez *et al*. 1982, Daly *et al.* 1988), excitable cells, such as neurons (Sonnewald *et al*. 1995, Bhakoo *et al.* 1996) and pancreatic islets (Danis *et al*. 1990, Papas *et al*. 1999). We have evaluated and extended a system derived from the perfusion system described by Gillies (Gillies *et al*. 1993, Pilatus *et al*. 2000) which involves growing cells on CultiSpher beads. We have replaced bioreactors with a simple flow-through culture tube, built from an NMR tube. The advantage of our system is that the simplicity allows us to harvest easily accessible cells for additional experiments following NMR culture.

Experiments in this laboratory investigate the relation between thiol redox modulation and therapeutic outcome, as applied to cancer (Livesey *et al*. 1988). Drugs, suitable for magnetic resonance detection, are supplied to superfused cells growing in culture in an

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NMR tube. The A549 cell line has been selected as a test system in which to measure their response to cytotoxic cancer therapy. This human lung adenocarcinoma line was chosen for its unusually high levels of glutathione (Kang *et al*. 1990), the most abundant low molecular weight thiol.

Monitoring the biological response to biochemical modifications that potentially alter therapy requires time intervals from hours to days. In order to influence the outcome of cytotoxic therapy, the modified condition must be maintained for a few hours. Cells are most responsive to cytotoxic therapy during exponential growth. Hence, a system that maintains exponentially dividing cells in culture for one to two days is required. Experiments to characterize cell cultures grown in NMR perfusion were undertaken and are reported here.

Our objective was to construct a system that cultured viable, growing cells in an NMR tube for spectroscopy extending over a few days. Specifically, this system of cells, growing on CultiSpher beads, needed to maintain actively dividing cells in exponential growth, contrasted with growth-arrested plateau phase, and adequate oxygenation for biologically valid results from the NMR data. In addition, we required that the cells be recoverable and viable for additional characterization at the end of the spectroscopy studies. The results, presented here, show that growth in the NMR tube, as assessed by cell counting after 1 and 2 days, compared well with the rate of growth in a parallel spinner culture. Furthermore, the rate of increase in ^{31}P-NMR detected metabolites paralleled growth as measured by cells/bead with no relative change in high energy phosphate resonances, indicating stable cellular energetics.

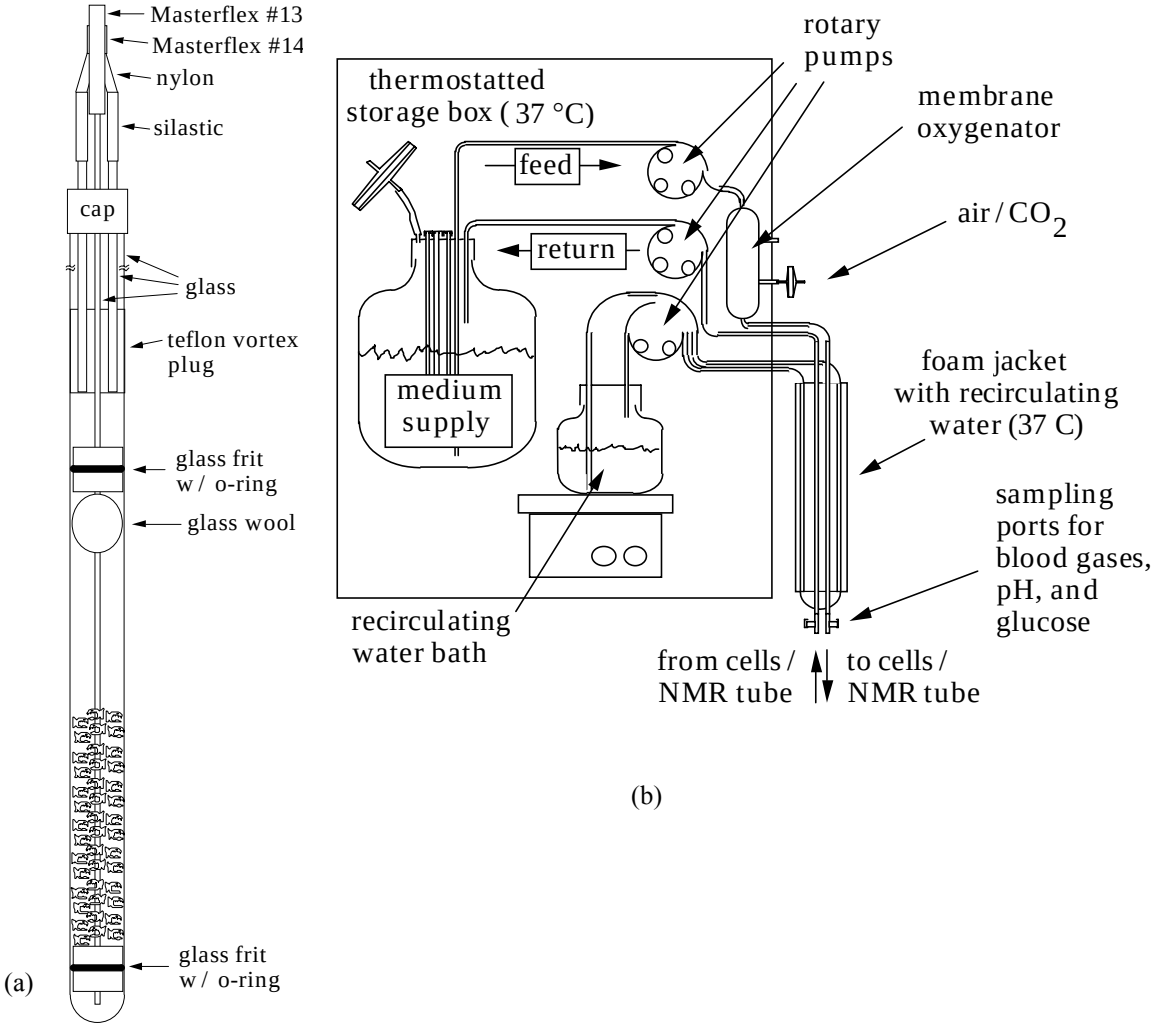

Fig. 1. *Schematic diagram of a) NMR perfusion chamber and b) perfusion system.*

Methods

Cell culture

The A549 human lung carcinoma was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in and perfused with 50 % Dulbecco's modified Eagle's medium and 50 % Ham F-12 nutrient mixture incorporating *l*-glutamine and 15 mM HEPES (Sigma Chemical, St. Louis, MO) supplemented with 10% (v/v) bovine calf serum (HyClone Laboratories, Inc., Logan, UT). 5 ml of a penicillin (10 000 U/ml)/streptomycin (10 mg/ml) solution in 0.9 % saline (Sigma) were added to 100 ml medium to help maintain sterility. A549 cells were grown in monolayer cultures in medium in 75 cm^2 Corning flasks. Cells were incubated at 37 °C with a gas phase of 5 % CO_2 in air at 100 % relative humidity. Under these conditions, this cell line has a doubling time of 21 hours. Cells were passaged weekly for no more than 50 passages.

Cells on CultiSpheres

A549 cells for seeding standard porosity, CultiSpher-G Macroporous Gelatin Microcarriers (CultiSphers, HyClone, Logan, UT) were grown to 80 % confluency in 75 cm^2 flasks. The CultiSphers were hydrated with sterile PBS for at least 24 h before being seeded with 2 x 10^7 cells in 100 ml medium for each 0.1 gram (dry weight) of beads. The cells were trypsinized (5.0 g porcine trypsin and 2.0 g Na_4EDTA/liter in 0.9 % NaCl (Sigma)) for 7 min at 37 °C in 5 % CO_2/air. The culture was fed with medium and maintained at 37 °C/5 % CO_2/air, spinning at 30 rpm for the first 24 hrs, after which the speed was increased to 35-40 rpm. One-half of the medium was replaced daily with fresh warmed medium, with samples removed periodically for assessment of growth. After about 90 h of growth, when the density of cells had increased from 80 to ~300 cells per bead, a portion of the culture was transferred to the NMR tube culture described below.

NMR perfusion chamber

The perfusion chamber was a 10 mm NMR tube fitted on the top with one inlet and two outlet tubes. These tubes were fed through a standard NMR cap to glass capillary tubes, which passed through a vortex plug (Fig. 1a). The exit tubes terminated at the lower end of the vortex plug, whereas the central feed tube was lead through a glass frit defining the upper boundary of the sample chamber. A reference capillary containing hexamethylphosphoramide (HMPA, 78 mM, relaxed with aqueous $Ni(NO_3)_2$) was glued to the feed capillary extending through the sensitive region of the NMR coil. A second glass frit was glued below the NMR probe's field of view to prevent recirculating detached cells and cellular debris from plugging the oxygenator. The sample chamber assembly (vortex plug, frits, reference capillary, and tubes) was removable to allow access to the region housing the cells. An additional piece of glass fiber was included above the cell-bead mass to further prevent particulates from obstructing the perfusion system. This entire assembly was gas sterilized with ethylene oxide after assembly.

NMR tube recirculation system

A 50 ml screw-top bottle, the reservoir for cell culture medium, was fitted with ports for supply (Masterflex #13, Cole-Parmer, Chicago, Ill.) and return (Masterflex #14) lines, gas exchange (through a 0.22 μm filter), sampling (Masterflex #13), and sample introduction. A dual-headed, peristaltic pump pushed medium through a pressure transducer to a membrane oxygenator (SETEC, Livermore, CA.), equilibrated with 5 % CO_2/air, at ~2-3 cc/min. The reservoir, pump, transducer, and oxygenator were contained within a thermostated cart (Fig. 1b), similar to Gillies *et al.* (1993). Supply and exhaust tubes were 3 meters long, allowing the reservoir and pumps in the cart to be a minimum of 1.5 meters from the base of the magnet. After assembly, this entire apparatus (reservoir, filters, oxygenator, and tubes) was autoclaved.

The supply and exhaust tubes were both pumped by a dual-headed peristaltic pump. The supply line was smaller in diameter than the exhaust line, to maintain a slightly lower pressure in the exit line. This had the advantage of maintaining a constant volume in the chamber. Supply and exhaust tubes were fed from the cart through a foam insulating jacket kept at 37 °C. The temperature was maintained by pumping water from a large reservoir, maintained at 37 °C, through a parallel set of Tygon® tubes enclosed in the same foam jacket. T-junctions at the top of the magnet were provided in both medium lines to allow sampling. pH, dissolved CO_2, and O_2 were monitored in the supply and exhaust lines to the NMR culture with analysis (Corning 170, pH/blood gas analyzer) of medium that was sampled with glass syringes. In early experiments, samples were taken every hour to verify the balance between inflow and outflow from the NMR culture chamber. Once it was determined

that this flow rate and gas exchange provided a good balance between supply and exhaust, sampling was reduced to twice daily. From the T-junctions at the top of the magnet, medium was pumped to and from the tubes attached to the top of the NMR perfusion chamber. The single exhaust tube was coupled to both outlet tubes from the perfusion chamber (top of Fig. 1a).

Prior to loading the perfusion chamber, the cells on beads were allowed to settle to the bottom of the spinner flask, transferred to a test tube, and rinsed 3-4 times with warm medium to remove cellular debris. Fifty milliliters of medium with suspended beads (equivalent to 50 mg dry weight) were transferred to each of two sterile NMR perfusion chambers equipped as described above. The remainder of the beads were maintained as a parallel culture in the spinner flask with fresh warmed medium in the proportion of 1 ml medium/milligram bead (dry weight). Samples were then removed from the spinner flask for staining, determination of cells per bead, and flow cytometry. NMR culture medium changes (100 ml) were accomplished daily by moving the entire recirculation system into a sterile, laminar flow hood to substitute a new reservoir with fresh warmed medium.

Glucose consumption

*F*or glucose consumption measurements, $7x10^7$ A549 cells grown on 100 mg of CultiSphers were removed from spinner culture and placed in an NMR perfusion chamber, as described above. The reference capillary in the chamber was replaced with one containing sodium 2-[^{13}C]-acetate (0.98 M, 99 % enrichment, Cambridge Isotope Laboratories, Woburn, MA) to provide a reference for NMR quantitation. These cells were then perfused with serum-free 50 % Dulbecco's modified Eagle's medium and 50 % Ham F-12 nutrient mixture. This medium was initially glucose-free for around 15 min during the tube assembly and its relocation to the spectrometer. 70 mg of 1-^{13}C-glucose dissolved in 3 ml of glucose-free medium were added through a 0.22 μm sterilization filter to the 50 ml of medium, resulting in 7.8 mM glucose. Acquisition of carbon-13 spectra was started immediately.

NMR spectroscopy

Phosphorous: Acquisition of 128 averages of ^{31}P spectra (10 kHz sweep into 4096 complex points) at 7 T was accomplished *via* a 5 second recycling of a 90° pulse (18 μs) followed by non-spinning acquisition with a commercial, thermostated, Bruker (GE-Omega) 10 mm probe at 37 °C. This provided relaxed spectra with a minimum signal-to-noise of 9 (unbroadened) or 30 (with matched exponential filter) for the resonances of choice (T_1's ~1.5 s). Integrals of 10 Hz broadened peaks were evaluated by fitting with VARPRO software (Van der Veen, *et al.* 1988). Carbon: proton-decoupled (MLEV-64), ^{13}C spectra (15 kHz sweep into 8192 complex points) at 7 T were acquired as 128 averages using a 3-second delay prior to a 90° pulse (27.5 μs) with the same commercial, thermostated, Bruker (GE-Omega) 10 mm probe at 37 °C retuned to 75.57 MHz. Temperature stability was confirmed *via* monitoring a thermocouple inserted into an NMR culture tube in the absence of cells while recirculating medium during a mock acquisition for 2 h. Non-spinning spectra were acquired with gated decoupling to minimize differences in nOe between glucose and lactate, but the intensities from the carbon spectra were corrected for differences in T_1 between glucose and lactate using the values found by Post *et al.* (1992). This provided serial spectra during uptake and metabolism of carbon labeled glucose which were broadened with a matched exponential filter before line-integration of transformed spectra.

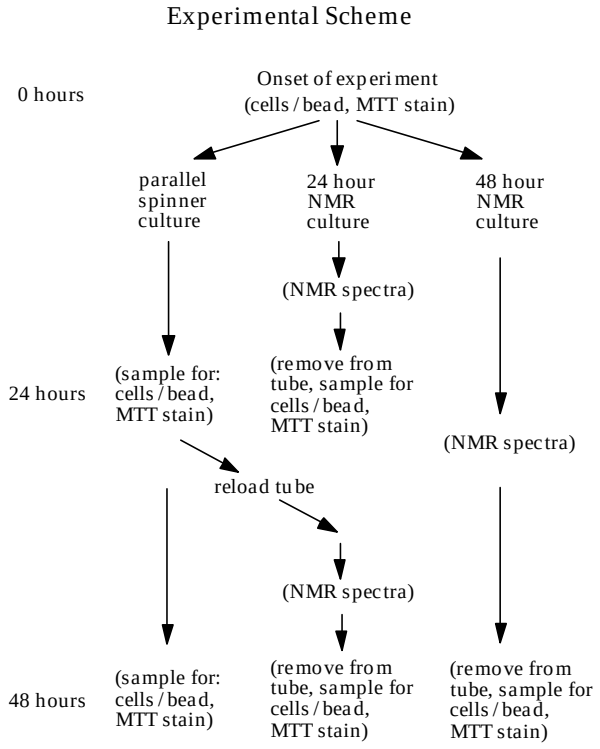

Fig. 2. *Schematic representation of experiment.*

Characterization of cells

Cell growth was monitored in NMR tube cultures and in the parallel spinner culture (Fig. 2). After 24 h, the beads were removed from one of the NMR tubes and replaced with an equivalent number of beads (50 mg, dry weight) from the spinner flask. The removed NMR culture and the parallel culture were then sampled for measuring cells per bead and cell cycle distribution by flow cytometry. After 48 h, beads from both of the NMR tubes were removed and sampled along with beads from the spinner flask. This entire protocol was repeated on three separate occasions.

Cells per bead

CultiSpher samples were withdrawn in duplicate 0.5 ml samples from the spinner flask or from resuspended NMR tube cultures, transferred to 60 mm bacteriological Petri dishes with a grid on the bottom, and counted with the aid of a dissecting microscope. Collagenase solution (2 mg/1 ml**,** Sigma, St. Louis, MO) was added and incubated on an orbital shaker for 1 h at 37 °C in 5 % CO_2/air. Cell clumps were broken up by drawing the solution through a 23-gauge needle. The sample was centrifuged at 3000 rpm, resuspended, and counted with a Coulter counter. From both of these determinations, an index of the number of cells per bead at a particular time after innoculation onto beads was obtained.

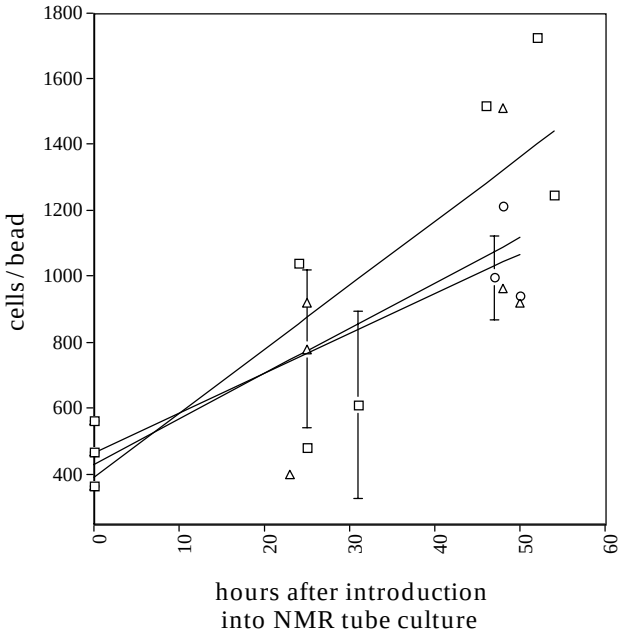

Fig. 3. *Measured cells/bead and linear fits for growth of A549 cells on CultiSphers: Δ - growth in NMR culture for 24 hours,* o *- growth in NMR culture for 48 hours, and - growth in parallel spinner culture for the duration. Error bars are the standard error of the fit for each of the three experiments.*

Flow cytometric analysis (Poot et al. 1991)

Cells harvested for flow cytometry were suspended in 10 mg/ml 4',6-diamidino-2-phenylindole (Sigma, St. Louis, MO) with 10 % DMSO and frozen at -70 °C. Samples were run on an ICP-22A (Ortho Diagnostic Systems, Rariton, N.J.) flow cytometer with UV excitation from a Hg source with a UG_1 excitation band pass filter and 400 nm UV cutoff filter. A minimum of 20 000 cells were analyzed to determine the fraction of cells in G_0, G_1 and S phase.

Results

Cell biology

Growth was monitored prior to introducing the cells into the NMR sample chamber to establish a consistent starting cell density for the experiments of nominally 300 cells per bead. Growth curves were compiled for cells grown on CultiSpher beads in spinner culture and in the NMR tube perfusion system by directly counting the number of cells per bead *versus* time. Flow cytometry was also monitored for each cell culture to determine cell cycle distribution, thus ensuring equivalent numbers of dividing cells. Cells recovered from the NMR perfusion tube were found to exclude trypan blue similar to the spinner culture, verifying that cell membranes were intact.

In initial experiments with cells growing on beads in the NMR culture system, single NMR tube cultures of between 100 and 150 mg of dry beads were used. The cells in spinner culture were allowed to grow for 7-9 days (resulting in 750-900 cells/bead) before transferring the culture into the NMR tube. This was longer growth and higher cell density than was used in subsequent studies with two tube cultures. This cell density and incubation time caused a marked pH decrease in the subsequent NMR culture. Decreasing spinner cultures to 50-75 mg starting bead mass provided NMR cultures with good physiological function and satisfactory NMR signal-to-noise ratio. The recirculating system then provided adequate superfusion, as inferred from the absence of pH change, at flow rates that did not disrupt the bead mass.

Table 1. Results from fit of growth data of spinner culture *versus* NMR tube culture.

Culture	Slope (cells/bead/hour)	r^2	Doubling time (hours)
Spinner	19	0.72	39
24 hour NMR tube	12	0.63	51
48 hour NMR tube	14	0.89	48

At the oxygen levels used in these experiments (100-200 torr), dissolved oxygen in the outflow was approximately 92 % of the input. To test what level of oxygenation could meet the demands of the cells, the input was intentionally reduced in one experiment to 50 torr and over one-half of the O_2 was extracted during one pass through the NMR tube. Thus, this comparison between input and output of dissolved oxygen provided another test of the sufficiency of the perfusion rate to preclude an oxygen gradient in the NMR tube cultures.

No decrease was found in the NMR signal from the lower starting conditions compared with the 100-150 mg cultures, since cells on 50 mg beads provided sufficient volume to fill the sensitive region of the NMR receiver coil. Further relief on the system was provided by lowering the starting cell density to ~300 cells per bead on the same 50-75 mg of collagen beads (~2.5 x 10^6 beads/gram dry weight). This population of cells, which was sufficient for NMR studies, helped to ensure exponential growth.

Growth data from cells that had been cultured for 24 and 48 h in NMR tubes and parallel spinner flasks are compared in Fig. 3. For each of three experiments, the average cell densities per bead were determined from each culture. Linear fits of the growth data were used for comparing the growth rates between the culture methods (Table 1). Within the scatter of the data, no differences were apparent between the NMR tube and spinner flask culture methods, validating similar growth rates in the two culture environments.

Cell cycle kinetics were measured by flow cytometry to monitor any changes in distribution of cells between G_0/G_1 and S phase which would result as the cells reached confluence in the NMR culture or their growth was otherwise limited by access to nutrients. Throughout each of the three experiments, the percentage in G_0/G_1 increased modestly (Table 2). This implies some crowding and decreased growth rate with increasing cells per bead. While crowding may explain the increase in G_0/G_1, the two culture environments compared well throughout the course of growth. This result establishes that the perfusion in the NMR tube culture provides adequate access to oxygenated medium comparable to the standard spinner culture. This was also implied by the lack of any pH gradient, which had been noted in the early, higher density experiments and was interpreted as hypoxia.

Table 2. Cell cycle distribution from spinner culture *versus* NMR tube culture.

		Spinner culture		NMR culture	
Exp	**Hours**	**Cells per bead**	**%G_1**	**Cells per bead**	**% G_1**
#	(after onset)				
	0	561	69	561	69
1	24	1041	70	800	73
	48	1518	79	995	75
	0	362	67	362	67
2	24	480	70	582	67
	48	1202	76	855	73
	0	270	70	270	70
3	24	448	79	646	85
	48	792	88	872	74

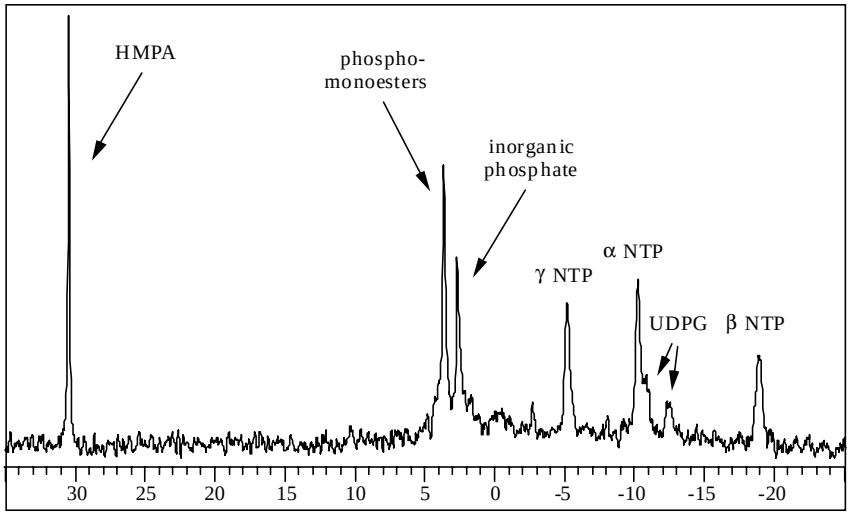

Fig. 4. *Typical ^{31}P A549 cell spectrum showing HMPA reference at 30.5 ppm. This spectrum represents a sum of 10 data sets 128 averages each (3 second recycle time) for a total of ~1 hour acquisition. The FID was preconditioned with 10 Hz of Lorentzian broadening prior to Fourier transform. Phosphorous linewidth on HMPA was ~6 Hz.*

NMR - ^{31}P spectroscopy

The predominant peaks in the ^{31}P spectra (Fig. 4) arise from nucleotide triphosphates (NTP), phosphomonoesters, inorganic phosphate and the reference HMPA resonances. The 3.68 ppm chemical shift of the phosphomonoester corresponds well with literary values for the chemical shift of phosphocholine (Daly *et al.* 1988, Kaplan *et al*. 1990, Ronen *et al*. 1991). However, since absolute identification has not been made for these cells, this peak shall be referred to as simply phosphomonoester (PME). Smaller peaks appearing near the α-NTP resonance are tentatively assigned to diphosphodiesters, such as uridine diphosphoglucose. The larger of the two peaks (nearer to α-NTP) also overlaps with resonances from NAD(P)H/NAD(P). With an initial cell density of ~300 cells per bead on 50-75 mg of CultiSpher beads, these ^{31}P metabolites were discernible after 128 scans (6 min), but integrals were analyzed for sums of 10 such spectra for a total data set corresponding to one hour each. Each spectrum was compared with the signal from the HMPA in the reference capillary to allow quantitative comparison between experiments.

The integrals from the three NTP peaks were summed to improve the statistics of the growth rate determination. To justify this combination, the integral of the γ-NTP resonance was plotted *versus* the integral of the β-NTP resonance. Since nucleotide diphosphate (β-NDP) overlaps with γ-NTP, but no corresponding γ-NTP with time might be expected if the energetics of of either oxygen or other substrates. Since the plot overlap occurs for β-NTP, an increase in the apparent yielded an r^2 of 0.91 and a 45° slope (1:1 correspondence), combination of the NTP peaks was justified.

The goal of the experiment was to establish that this NMR tube culture system was biologically valid. This was further supported by a comparison between growth as the number of cells counted per bead and growth as the increase in NMR signal intensity. Plots of either PME or NTP integrals (related to HMPA) *versus* cells/bead yielded good correlations and similar slopes (Fig. 5). Therefore, using either of these NMR integrals as an index of growth is valid.

The lack of a creatine phosphate peak (PCr), an often observed high energy phosphate constituent, hinted at compromised cellular energetics. Concern over the absence of this compound, which buffers ATP consumption during work in many cell types, lead to assay of the cell line for creatine kinase (CK), the enzyme responsible for facilitating this buffer function. The clinical assay on 1.6×10^7 cells resulted in CK activity below 5 units/l, which was the detectable limit of the assay. The lack of a PCr peak, therefore, reflects that essentially no detectable CK activity is present; the spectra, as shown, are typical for a healthy, fully functional A549 cell line.

^{13}C spectroscopy

In addition to establishing a perfusion system for the growth of cells, preliminary data was obtained to show the utility of the system for monitoring cellular metabolism. A simple glucose consumption measurement was made for a culture of cells superfused with 7.8 mM [1-^{13}C]-glucose. Using a reduced perfusion volume, a

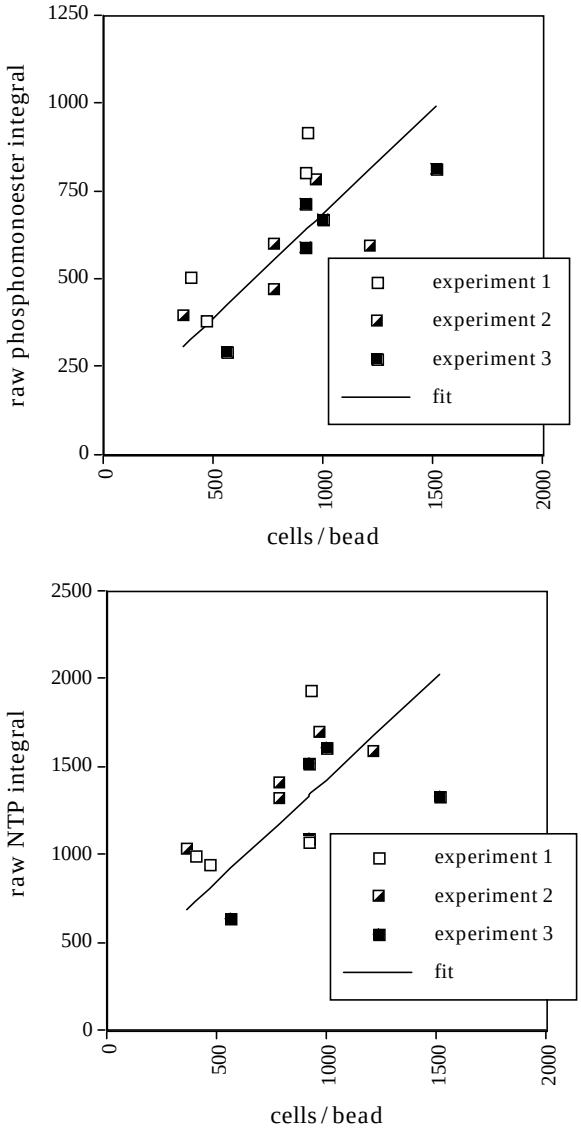

Fig. 5. *Comparison of growth for A549 cells as assessed by cells/bead and NMR metabolites: a) phospho-monoester integrals versus cells/bead (slope = 0.593(0.006), intercept = 92(65), and r^2 = 0.79) and b) NTP integrals versus cells/bead (slope = 1.16(0.04), intercept = 270(156), and r^2 = 0.71).*

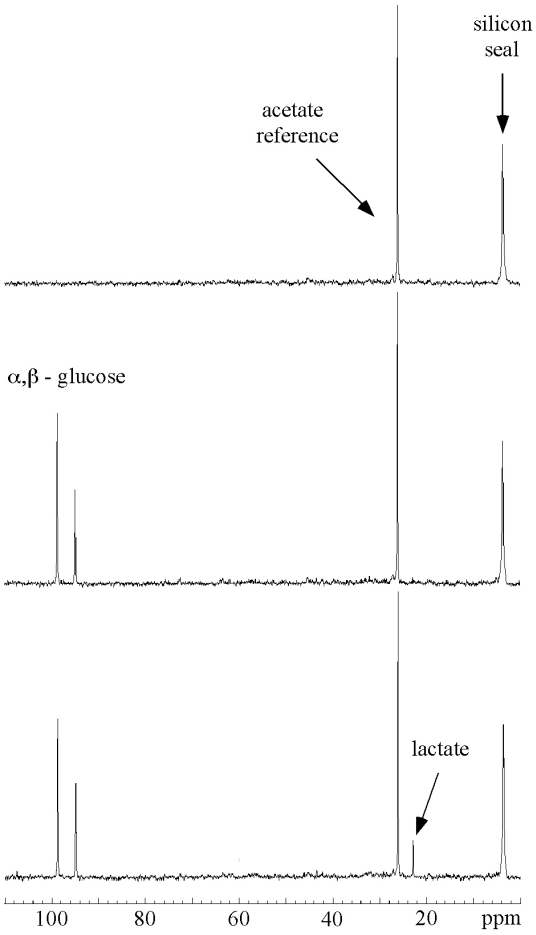

Fig. 6. *Carbon spectra from A549 glucose consumption measurement. The top spectrum is the cell culture prior to glucose infusion with peaks from the sodium 2-^{13}C-acetate reference (26.1 ppm) and the silicon sealant used to mount capillary (3.5 ppm). The middle spectrum is the second acquisition after glucose infusion with new peaks from α- and β- $^{13}C_1$-glucose (98.7 and 94.9 respectively). The bottom spectrum is after 3.75 hours of recirculation of glucose labeled medium with an additional peak at 22.9 ppm from lactate produced during the experiment.*

high signal-to-noise response obtained (Fig. 6) from the substrates and products, allowing the use of line integrals to quantify the predominant metabolic species: namely α– and β-glucose (98.7 and 94.9 ppm) and lactate (22.9 ppm) (Post *et al.* 1992). Indeed, the lack of additional resonance, present in the culture, established lactate as the predominant metabolic product from glucose consumption. The intensities *versus* time for the glucose supplied, the lactate produced, and their sum were plotted (Fig. 7). From this time course and a count of the cells, a glucose consumption rate of 0.25 μmol/min/10^8 cells was obtained. This compares well with literary value for the CEM cell line (a human leukemic cell) of 0.26 μmol/min/10^8 cells which was obtained by a similar NMR observation (Post *et al.* 1992). At this lactate production rate, lactate concentration in the normal, larger perfusate volume would be only 5 mM in 24 h, further underscoring the

sufficiency of the growth conditions. Given the lack of additional metabolite peaks, it is reasonable to ascribe the decrease in the sum of glucose and lactate to the loss of labeling carbon dioxide. The CEM cells were not cultured in the tube, but rather were suspended from a pellet by bubbling air through the NMR sample tube. Though these cell lines may not be expected to show similar rates, the value obtained here is at least reasonable and shows the feasibility of such measurements with this experimental setup.

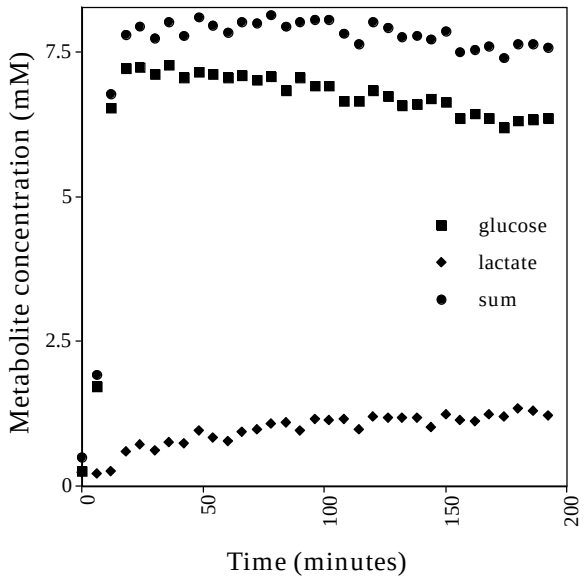

Fig. 7. *Glucose consumption measurement for A549 cells supplied with 7.8 mM 1-^{13}C labeled glucose. Acquisition begins at injection (time = 0) of 3 ml of glucose solution (70 mg [1-^{13}C] - glucose/3 ml) into glucose-free medium.*

Discussion

These studies validate a simple NMR tube culture system in the context of these experiments. With the established minimum cell density of ~300 cells per bead on 50-75 mg of collagen beads (or ~3 x 10^7 cells in the NMR culture) typical ^{31}P metabolites (NTPs and PME) are easily detectable for quantification. Further, the good correlation between cells per bead and NMR peak integrals during growth serves both to confirm this system as a biological model and to provide an *in situ* index of health of the system for subsequent experiments. Potentially, changes in integrals of PME or NTPs with time (deviation from control doubling times) could be used to infer energy utilization in response to treatment. Using the external reference in the NMR spectra will facilitate cross-comparisons of data sets for monitoring other labeled substrates that might modify the response to cytotoxic treatments.

Utilizing ^{13}C NMR of labeled metabolites, such as glucose in these studies, allows monitoring of relative changes in key metabolic pathways. For example, since the signal from intracellular TCA and glycolytic intermediates is low, a decrease in the C_1 position of glucose can be reasonably ascribed to conversion of glucose to lactate or to production of CO_2 *via* either cleavage in the pentose phosphate pathway or through multiple turns of the TCA cycle. Therefore, since the pentose phosphate pathway is responsible for generating reducing equivalents to provide protection of cells from oxidative insult, treatment of cells with cytotoxic drugs will result in increase in label loss. Similarly, this pathway is important in providing key intermediates for growth, so that decreases in label loss would be consistent with slowing of growth. Though previous studies have begun to look at these potential uses, this system provides a better biologically stable environment for monitoring cellular metabolism.

The independent criteria provided by routine biological evaluation of growth as cells per bead and cell cycle kinetics from flow cytometry show that this system parallels the spinner culture. The ease of recovery of the cells from the NMR culture will allow assessment of plating efficiencies and other characteristics of cultures treated *in situ* in the NMR culture. These same growth comparisons will be made for cultures which have been treated to modulate thiol/disulphide equilibria. Upon treatment (chemo- or radio-therapy) of these cells (with or without modulation of thiol/disulphide equilibria), NMR measurement of growth will establish the importance of the protective effects of thiols involved in different approaches to cancer therapy.

Acknowledgements

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