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A ^{1}H spin echo NMR study of the HeLa tumour cell

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Phosphorylcholine, phosphorylcreatine, lactate, glutathione, glycine and leucine were identified in living HeLa cells in the first study of this cell type by ^{1}H spin echo NMR spectroscopy. The technique has the advantage that it is non-invasive, providing detailed structural information on individual species present in the cell matrix. It has been used in this case to study the rate of energy consumption following the activation of the glycolytic pathway with glucose.

Glycolysis; HPLC; Cancer; Spin echo; ^{1}H-NMR; (HeLa cell)

1. INTRODUCTION

Spin echo NMR spectroscopy is a non-invasive probe which is now an accepted method for the study of cellular metabolism in intact erythrocytes [1]. The technique is selective and provides detailed structural information for individual metabolites in the living cell of which changes in the glutathione:glutathione disulphide ratio [2,3] and dehydrogenase activity [4] are good examples. If the technique can be extended to specific leukocyte fractions, more precise methods for studies of disease processes and drug metabolism could be achieved. One study of the differentiation of the erythroid Friend leukemia cells has been reported [5]. We report here the use of spin echo NMR spectroscopy with the malignant HeLa cell as a first example of a non-erythroid cell type. The kinetics of the energy consumption by this cell type is detailed and discussed.

2. EXPERIMENTAL

HeLa cells were grown in enriched RPMI 1640, the composition of which was RPMI 1640-Hepes (500 ml), (1%, w/v) glutamine (5 ml), 7.5% sodium bicarbonate (2.3 ml) and 10% FBS (50 ml). Cells were grown in F-120 flasks at 37°C for 5–6 days before splitting. The growth curve was shown to plateau at 7 days. They were harvested by centrifugation at 1500 × *g* for 10 min after removal from the glass by the addition of trypsin solution (10 ml of 2.5% trypsin solution to 90 ml buffer solution consisting of 6 g NaCl, 6.6 g trisodium citrate and 0.01 g phenol red in 1 l distilled H_2O). Cell viability was checked by harvesting the cells as above and resuspending in sterile physiological saline at 0.5×10^6 cells in 3 ml. To 0.2 μl of this suspension was added 0.2 ml of trypan blue stain and the percentage of live cells counted using a haemocytometer. The cells were found to remain viable in physiological saline for at least 5 h (>90% viability) at room temperature.

The cells were harvested, washed twice [it has been reported that excessive washing of the HeLa cell with physiological saline causes cell lysis [11]. We have found two washings with ^2H_2O/NaCl sufficient to remove excess media and provide a deuterium lock for the spectrometer, while not causing excessive lysis.] in a minimum amount of D_2O/NaCl (0.154 M) and packed into a previously autoclaved 5 mm NMR tube. The standard sample

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size was approx. 10^9 cells in a total volume of 0.4 ml. A Bruker WM 250 MHz Aspect 2000 spectrometer was used to record all spectra. The spin echo spectra were recorded using a Hahn spin echo pulse sequence ($90-\tau_2-180-\tau_2$).

Cell lysates were prepared by the sonication of approx. 10^7 cells in 1 ml H_2O/NaCl (0.154 M). Debris was removed by filtration. HPLC separation of the lysates was carried out on a 250 × 4.6 mm i.d. column slurry packed with ODS Hypersil (5 μm) (Shandon Southern). Elution was isocratic, the eluant being methanol/water/40% (w/w) tetrabutylammonium hydroxide (940:60:1) with the pH adjusted retrospectively to 3.5 by the addition of 10% (w/v) phosphoric acid. The elution was at ambient temperature at a flow rate of 0.8 ml/min. Detection was by a Waters 490 multiwavelength detector set at 220 and 254 nm. The chart speed was 5 mm/min. A typical chromatograph of a cell lysate is shown in fig.1.

3. RESULTS AND DISCUSSION

NMR spectra obtained from the HeLa cells are shown in fig.2. The normal FT NMR (trace a) shows resonances which arise from the cell membrane, protein and solute molecules. Applying the spin echo pulse sequence filters the large molecules from the spectrum by virtue of their shorter relaxation times. Thus, the spin echo spectra (fig.2b–e) represent only the small resonant cytosolic components of the cell. A series of spectra are shown (τ_2 = 30, 50, 60, 70 ms) since the technique relies on relaxation times and important molecules could have a null point at a particular delay time (τ_2) and therefore would not be seen in the spectrum.

The resonances (fig.3) have been assigned on the basis of reported information [6,7] and by the procedure of standard addition to the cells both as whole cells in the spin echo NMR and as lysates in the HPLC. Glycine [7] has been shown to be a ma-

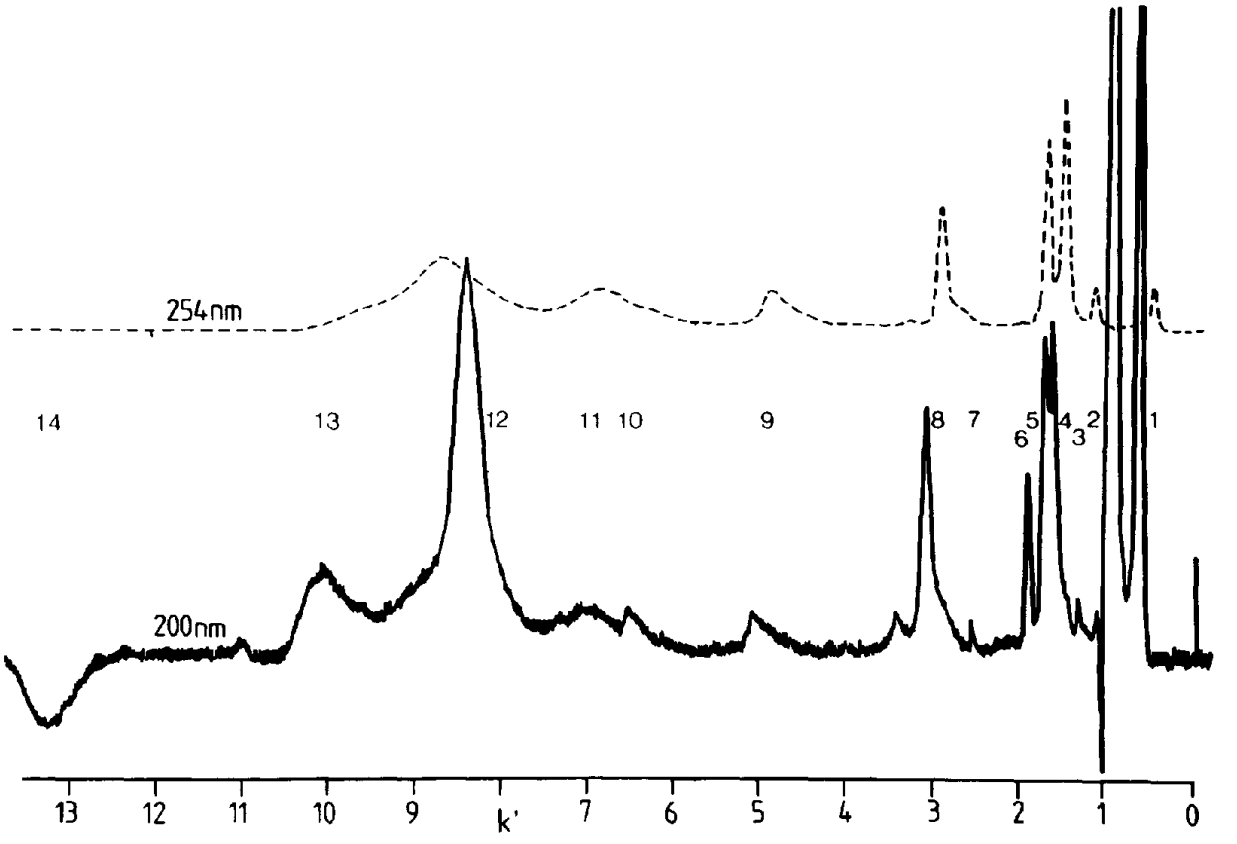

Fig.1. A typical HPLC chromatograph of a HeLa cell lysate.

Fig.2. Normal 250 MHz FT NMR (a) and spin echo NMR (b–e) of the HeLa cell; (b) τ_2 = 30 ms; (c) τ_2 = 50 ms; (d) τ_2 = 60 ms; and (e) τ_2 = 70 ms. Each spectrum consists of 1000 scans on a total sample size of 10^9 cells/0.4 ml ^2H_2O/NaCl (0.154 M). All spectra were recorded at 20°C. A small presaturation pulse was applied to the water resonance prior to accumulations.

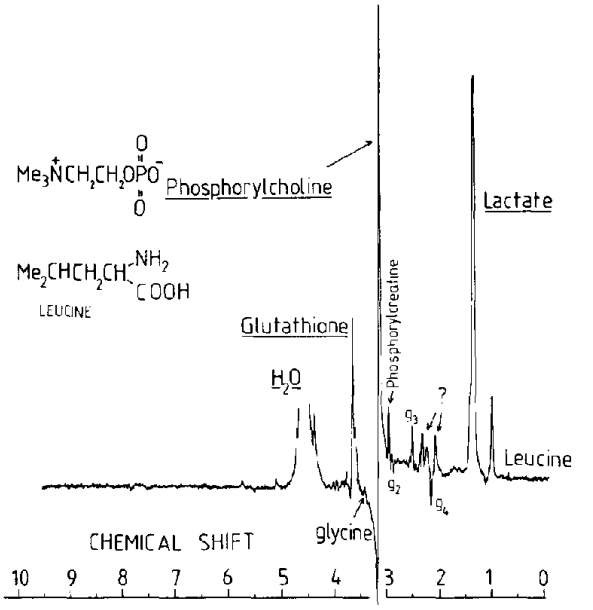

Fig.3. ^1H spin echo NMR of the HeLa cell; τ_2 = 60 ms.

jor constituent of the free amino acid pool (table 1) of the HeLa cell and is easily observed at τ_2 = 30 ms. Glutathione and leucine are observed to the exclusion of other cellular components (table 2) because these molecules have intense resonances, namely the glycyl methylene of glutathione and the methyl resonances of leucine. Other major components such as phenylalanine, tyrosine and histidine are not observed because these species have no intense resonant groups in the spin echo NMR as used in this case. Considerable phase modulation is seen in the lactate, and to a lesser extent the leucine signals. The signal intensities diminish as the delay time (τ_2) is increased. Phosphorylcholine and phosphorylcreatine as constituents of the cell go largely undetected using a UV detector with HPLC separations. These assignments are made on the basis of standard additions to the whole-cell spin echo NMR and on the basis of the previously reported ^{31}P NMR [8]. The latter study indicates appreciable concentrations of both these molecules, and it is reasonable to expect that in these concentrations they will appear as prominent species in the ^1H spin echo NMR also. Furthermore, the assignments of the peaks to these molecules is consistent with the whole cell study of Agris and Campbell [5] and the lysate study of Evanochko et al. [9] both of which have identified

Table 1

The relative concentration of the amino acids in the cytosolic pool of the HeLa cell [7]

Amino acid	Concentration (nmol)
Glycine	11.9
Alanine	6.81
Leucine	6.34
Valine	5.44
Lysine	4.18
Phenylalanine	3.30
Isoleucine	3.22
Tyrosine	3.00
Arginine	2.37
Histidine	2.17

Table 2

Peak assignments for the ^{1}H spin echo NMR of the HeLa tumour cell

Compound	HPLC retention time		^{1}H NMR chemical shift referenced to the glycyl function, function of glutathione as 3.75
	Peak no.	k	
Glutathione			
reduced	13	10	3.75, 2.8, 2.6, 2.4
oxidised	12	8.7	
Glycine	unretained		3.5
Phosphorylcholine	unretained		3.25
Phosphorylcreatine	unretained		3.0
Lactate	unretained		1.4
Leucine	4	1.6	1.1
Tyrosine	5	1.5	unobserved
Glutamine	6	1.9	unobserved
Phenylalanine	8	3.2	unobserved

these molecules in their tumour lines. Evanochko et al. have further identified these particular molecules using an infrared detector with an HPLC separation. Study of cellular metabolism by NMR methods will depend on the ability to maintain cell viability. For these initial experiments it was decided to suspend the cells solely in D_2O saline and to estimate the viability under these conditions ($t > 5$ h). This test is quite stringent for a cell in that much more complex nutrient media are usually used to increase lifetime but it does give a conservative estimate for the time scale in which experiments can be done with viable cells.

Cancer cells derive the bulk of their biochemical energy from the production of lactate rather than the complete degradation of glucose via the tricarboxylic acid cycle and consequently the kinetics of lactate production can be used to assess the energy status of the cells in the culture or tumor following glucose addition. Added glucose (2.0 mg/0.4 ml packed cells) appears as an asymmetric doublet at 5.0 (fig.4) in the spectra. The doublet arises as a result of the two glucose anomers (α,β). The upfield line is assigned to the α anomer. The HeLa cell has a definite preference for the α anomer, behaviour previously reported by Shulman et al. in ^{13}C NMR experiments with micro-organisms [10]. The sugar is metabolised efficiently to produce a variety of products, dominant amongst these being lactate (δ 1.5). The behaviour of the β anomer is complex. The small reduction in its concentration is likely to arise from a combination of mutorotation ($\alpha \rightleftharpoons \beta$) and, if it exists, reduced metabolic activity. Using the signals from α glucose and lactate the glycolytic process can be monitored as a function of time (fig.5), the rate of cellular uptake of sugar and production of lactate can clearly be seen to be linked. The lag phase between the maximum rates of uptake and biosynthesis is consistent with the chain of biochemical events involved in the overall reaction.

The pulse sequence used to obtain spin echo spectra modulates the intensities of the various lines. Thus signal intensities in the spectra, while still reflecting the individual species concentrations, are no longer reliable for direct comparison without prior calibration. Using standard mixtures of glucose and lactate (lithium salt, monohydrate), the intensities of the important lines were measured in the normal FT NMR experiment (ratio 2:3.04, glucose to lactate) and compared with those obtained in the spin echo for the same sample (ratio 2:4.21). The degree of modulation experience between the signals calculated above can be transferred to the in vivo experiment only on the addition of a precise amount of either species, in this instance 2.0 mg glucose, as an internal calibrant.

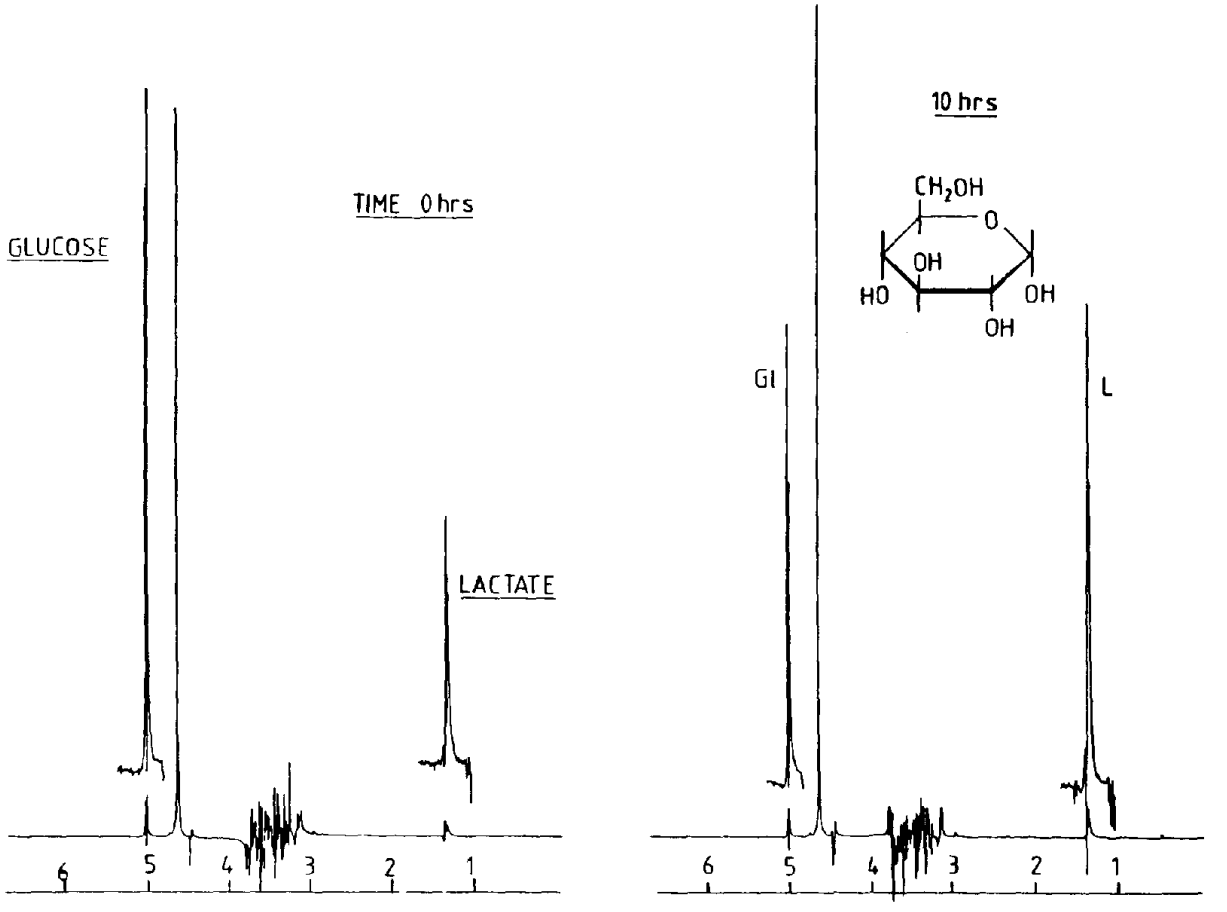

Fig.4. ^{1}H spin echo NMR of the HeLa cell (τ_2 = 60 ms) at time t = 0 h and t = 10 h, the medium being supplemented with 2.0 mg glucose.

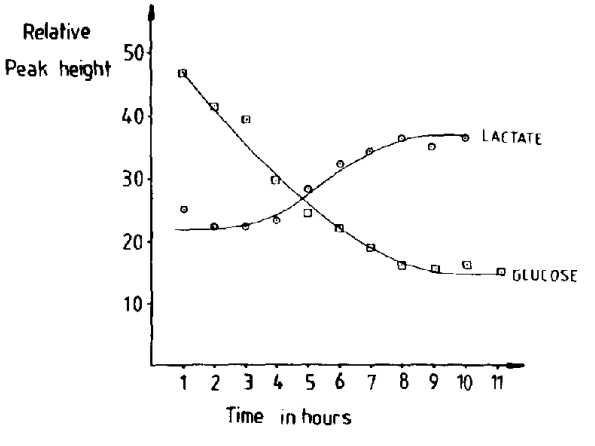

Fig.5. The time course of glycolysis in the HeLa cell as depicted by the observed changes in the concentrations of α glucose (at δ 5.2) and lactate (δ 1.5).

Using this approach, an estimate of the amount of lactate being produced by the HeLa cell can be made; $3.3 \pm 0.3 \times 10^{-6}$ mol/10^{10} cells. Lactate production, however, reaches a maximum rate of $9.2 \pm 0.6 \times 10^{-11}$ mol/s some 5 h after substrate addition. Considering the basic stoichiometry of lactate production (eqn 1),

$$\text{Glucose} \longrightarrow 2 \text{ lactate} \quad G = -196.4 \text{ kJ/mol} \quad (1)$$

the turnover (yield) can be calculated at 14.8%, with a total free energy change, due solely to lactate production, of -2.9 kJ/mol for the culture (10^{10} cells).

Thus, the combination of HPLC and ^{1}H spin echo NMR for analysis of cell composition and analyte identities is valuable, both because they provide complementary information in some cases

and because the NMR technique has the advantage that chemical processes in the living cell can be maintained in situ. The techniques have different sampling requirements since large cell numbers are required for NMR whilst HPLC is sensitive. In combination this allows monitoring of intact cell metabolism and more complete investigation of subfractions.

The ^{1}H spin echo NMR method is clearly a very powerful one in that it enables specific metabolites to be monitored in the living cell without the need for the addition of probe molecules or high-energy sources which can alter the chemical processes. It is therefore ideal as a model system for the study of the action of drugs and natural product uptake by the whole cell. Each cell type is likely to be different in both its composition and the rates of various metabolic processes. The HeLa cell is one stable and well-defined type which is sufficiently viable to enable experiments of a few hours to be performed readily, as our study of the rate and nature of energy consumption on activation of the glycolytic pathway with glucose illustrates.

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