HUMAN ERYTHROCYTE METABOLISM STUDIES BY ^{1}H SPIN ECHO NMR

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1. Introduction

On account of their ready availability and comparatively simple cell physiology, red cells have been studied more intensively than any other mammalian cell system [1]. These studies have involved a wide range of physical and chemical techniques.

Within the last four years, NMR has been added to this list of techniques by the use of both ^{31}P [2,3] and ^{13}C [4] in studies of whole red cells. NMR has a unique advantage over most investigation procedures in that it allows a non-invasive inspection of even the most delicately balanced system. This property has been used to advantage in studying a range of complex, intact systems such as muscle [5], bacteria [6,7] and chromaffin granules [8,9].

The limited distribution of a nucleus such as ^{31}P relative to ^{1}H within biological systems has, until recently, made it advantageous to trade the lower sensitivity of the ^{31}P nucleus for the much greater spectral simplicity. The ubiquity of the hydrogen nucleus in biological systems combined with the non-selective nature of the NMR method usually leads to a broad undecipherable envelope. However, the recent application of spinecho methods [9–11] in NMR has greatly increased the possibilities of the ^{1}H experiments by providing a means of selecting the resonances observed on the basis of spin–spin relaxation time, which is in turn related to the mobility of the residues or molecules involved.

In the work presented here, a few of the interesting features associated with whole red cells are studied by this new technique. The aspects on which we have concentrated have already been studied by other techniques and include

(a) The measurement of internal cell pH.
(b) The time course for changes in the GSH-GSSG status in the cell.
(c) A brief study of glycolysis in H_2O and D_2O.

2. Experimental

The red cells were prepared from freshly drawn venous blood by washing twice with isotonic saline (0.154 M) in H_2O, twice with isotonic saline in D_2O and twice with Krebs Ringer [12] in D_2O. In this state the cells were fully depleted in sugars and lactate but the glutathione was mostly in the reduced form.

For comparative studies, a portion of the red cells was washed with H_2O solution.

The spectra were obtained on a Bruker 270 MHz FT spectrometer with quadrature detection using a $90°-\tau-180°-\tau$ sequence of pulses and delays [10]. The delay time, τ, which was a controlled

Abbreviations: NMR, Nuclear Magnetic Resonance; DSS, 2-2 dimethyl-2 silapentane-5-sulphonate; GSH, reduced glutathione; GSSG, oxidised glutathione; FT, Fourier transform

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Table 1
Chemical shifts and assignments in the spectra from erythrocytes

Peak designation	Figure in which peak is annotated	Chemical shift (ppm) with respect to DSS	Assignments: position in molecule(s) to which ^1H attached
a1	2	8.49	C(8) of purine } of, e.g., ATP, ADP, AMP
a2	2	8.15	C(2) of purine } of, e.g., ATP, ADP, AMP
a3	1b, 2	6.95	C(1′) of ribose } of, e.g., ATP, ADP, AMP
g1	2	3.76	αC of Gly
g2	2, 3b	2.92 (at centre of gravity)	S linked C of Cys } residue of glutathione
g3	2, 3a	2.55	γC of Glu
g4	2, 3b	2.15	β C of Glu
h1	2	8.22	C(2) of His
h2	2	6.97	C(4) of His
h3	2	3.20	–
h4	2, 3a	3.02	– } residues of haemoglobin
h5	2	2.97	–
h6	2	~1.2 – ~1.5	C of methyls split by adjacent CH, e.g., Val
^1D	3a	1.26	C(3) with C(2) deuterated } of lactate
^1H	3b	1.32	C(3) with C(2) protonated } of lactate
p1	3b	2.36	C(3) with C(2) unhydrated } of pyruvate
p2	3b	1.44	C(3) with C(2) hydrated } of pyruvate
u1	2	4.07	Unassigned
u2	2	3.91	Unassigned
u3	2	3.54	Unassigned
u4	2, 3b	3.24	Unassigned, but coincident with quarternary amine methyls of acetylcholine
w	1a,b,c	~4.3 – ~4.8	Water

variable was normally set to 60 ms. This sequence was repeated at 1 s intervals and 300 transients were normally accumulated.

The time course experiments were run in a 5 mm coaxial NMR sample tube with an external DSS marker. Usually the sample tube was spun during the experiment. The spectral amplitudes were normalised to the peak u4 in the spectra (see table 1). The red cell samples in H_2O were also run in coaxial tubes with external D_2O to provide a lock signal. The large water peak was reduced by a selective suppression pulse [13] applied to the samples at all times other than during data acquisition (the acquisition time was 0.52 s).

In all of these experiments, the packed red cells were re-suspended in about 25–40% of their volume of buffer solution as this provided a liquid of manageable viscosity while minimising the sedimentation rate of the red cells over the duration of the experiment. In these experiments, no noticeable sedimentation of the cells was observed.

3. Results and discussion

The power of the spin echo technique to extract relatively sharp lines from the many overlapping lines in the red cell spectrum is illustrated in fig.1.

Figure 1a shows the normal spectrum which is dominated by resonances from haemoglobin. With a 20 ms delay the same sample gave the spin-echo spectrun shown in fig.1b where the glutathione spectrum with some of its fine structure now becomes apparent. At this delay time, the C(2) His resonances from haemoglobulin can be seen sufficiently clearly to determine the internal pH of the cell to within 0.03 pH unit using the pH titration curves of Brown and Campbell [11] as a template. The external pH for this sample

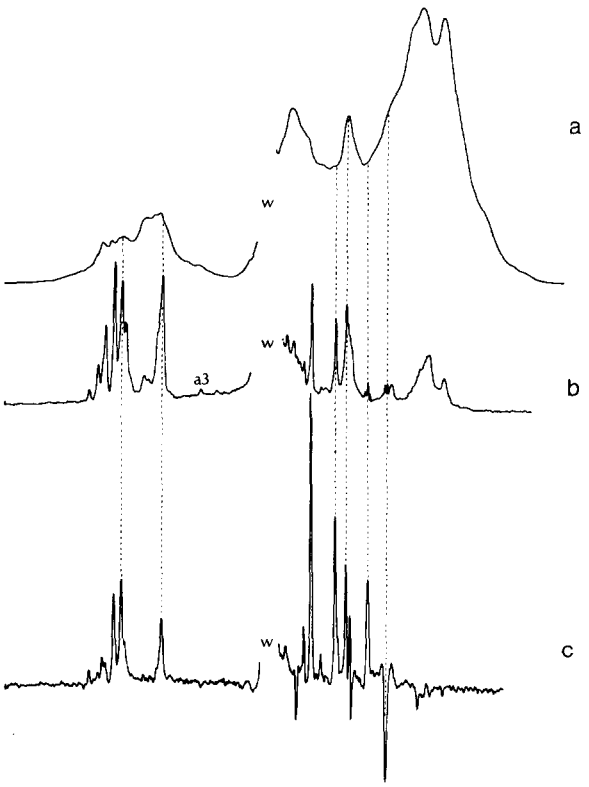

Fig.1. 270 MHz ^1H spectra of glucose depleted red cells at 37°C. (a) Normal Fourier transform spectrum. (b) Spectrum obtained using a $90°-\tau-180°-\tau$ spin echo sequence, with τ = 20 ms. (c) Spin echo spectrum with τ = 60 ms.

was measured by pH electrodes to be 7.46 whereas the internal pH from the haemoglobin is 7.40 at 37°C.

Using a 60 ms delay, the sharp resonances from mobile species present in millimolar concentration can be seen in fig.1c, and some assignments are given in table 1. These assignments were checked by comparison with previous work [14] and by comparing model compounds.

More detailed assignment was made by studying spin-echo spectra as a function of τ and by spin-echo double resonance [5]. These methods depend on the fact that when a peak is split into a multiplet by homonuclear coupling, its phase in the echo spectrum is changed according to the nature of the multiplet and the length of the delay before the echo. Figure 1c and fig.2 show several coupled glutathione peaks with a phase different from the main bulk of the spectrum. Not only are such phase changes diagnostic in themselves, but their variation with delay time can be used to separate overlapping peaks. Spin-echo resonance involves irradiation of a particular resonance during the pulse-delay sequence as this changes the multiplicity of the resonances coupled to the irradiated peak thus changing the phase of the coupled peaks in the spin echo spectrum.

This type of approach has provided some of the assignments shown in table 1. A few of the peaks

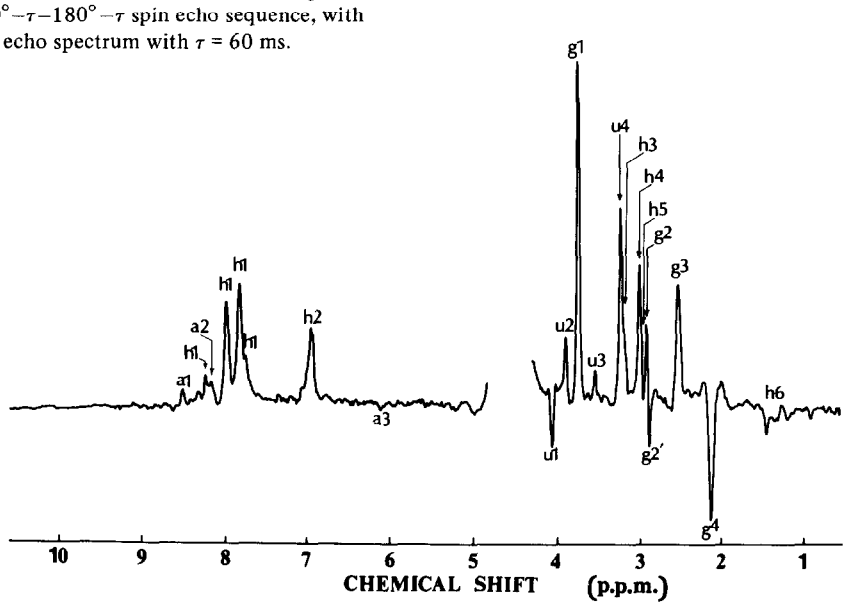

Fig.2. Expanded version of fig.1c with assignments (see table 1 for an interpretation of the symbols).

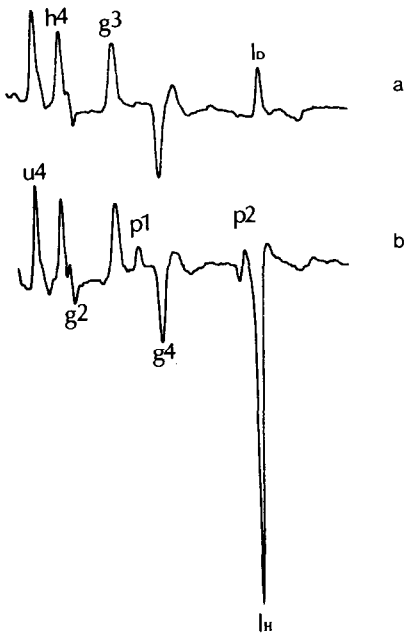

Fig.3. (a) Spin-echo spectrum (τ = 60 ms) of red cells incubated for 2 h in D_2O with 10 mM glucose. The lactate methyl resonance is upright. (b) Spin-echo spectrum (τ = 60 ms) of red cells incubated for 2 h in H_2O with 10 mM glucose. The lactate methyl resonance is larger and of opposite phase to the resonance in fig.3a.

which occur in these spectra vary greatly from one preparation to another in terms of their relative intensity (e.g., all the u-peaks). Some variation is to be expected from changes in the diet and condition of the donors but no systematic correlation has yet been attempted.

Figure 2 shows a spectrum from red cells in the sugar depleted state, so that no lactate is observed. Table 1 combines the assignments indicated on all the spectra.

In fig.3, the same stock of red cells has been incubated for 2 h with 10 mM glucose. The sample used for fig.3a was prepared in D_2O and for 3b in H_2O.

Two points are immediately obvious. The first is the difference in phase of the lactate methyl resonance. This arises from differences in the C(2) substituents of the lactate molecule. When the lactate dehydrogenase (EC 1.1.1.28) converts pyruvate to lactate, it transfers a hydrogen atom from NADH to the C(2) carbon of pyruvate. This hydrogen is spin coupled to the lactate methyl causing the resonance to be inverted at τ = 60 ms (fig.3b). In the D_2O solution, the glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) transfers a deuterium atom to NAD^+ to give NAD^2H and this deuterium atom is ultimately transferred to the lactate [16] where the heteronuclear coupling produces no changes in phase. It should be mentioned that the first time through the NAD^+–NADH–NAD^+ cycle, it is the hydrogen from the 4A position on the reduced nicotamine ring, rather than the newly acquired deuterium, which is stereospecifically transferred to the lactate [17]. Thus even in 100% D_2O, glycolysis should generate initially an amount of lactate with C(2) hydrogen equal to the sum of the concentration of NAD^+ plus twice the concentration of NADH present in the system at the start, provided that there is no significant utilisation of NAD^+/NADH elsewhere.

The other obvious feature of fig.3 is the difference is size of the lactate peaks in the two cases. Apart from any differences in the rate of glycolysis in D_2O solution relative to H_2O, the cause of this difference in peak intensity is due to the substituents on the methyl carbon. Solvent exchange at the triose phosphate isomerase (EC 5.3.1.1) and aldolase (EC 4.1.2.7b) steps will ensure that in D_2O about two thirds of the lactate will be present in the form $CD_2H.CDOD.COOD$ and about one third, $CH_2D.CDOD.COOD$ [16].

The time course for the rate of production of lactate is easily followed using such spectra as shown in fig.4. Two time courses have been followed simultaneously. A 1 ml sample of red cells in D_2O buffer, depleted in sugars, was titrated with *tert*-butyl hydroperoxide till all the glutathione was in the oxidised form [18]. In the sugar-depleted state the cells were metabolically dormant and no recovery of the glutathione was observed till glucose was added. Sufficient glucose was added to give a concentration of 10 mM, the sample thoroughly mixed and the timecourse then started. During the timecourse, the lactate concentration was monitored by the lactate methyl resonance. The reduced glutathione was monitored by the negative excursion of the cysteine methylene resonance, peak g2′ (this peak is positive and in a different position in the oxidised form) and by the intensity of the glycine methylene resonance, g1 (the intensity of this peak is empirically observed to

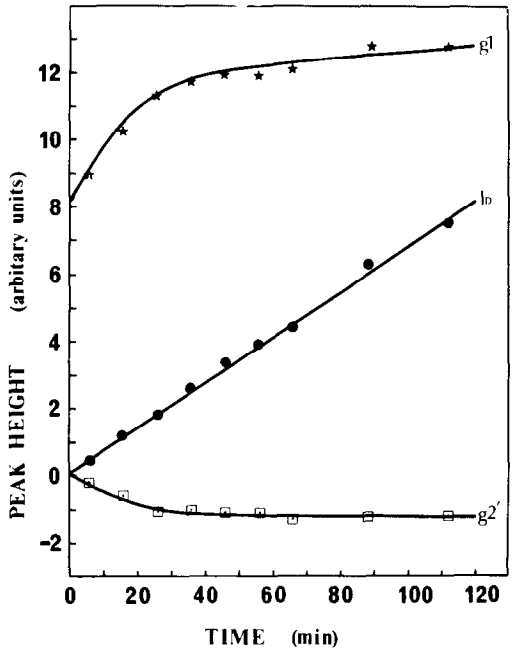

Fig.4. Time course for the production of lactate and the reduction of glutathione after the addition of glucose to red cells in which the glutathione had been oxidised with *tert*-butyl hydroperoxide.

decrease in the oxidised form relative to the reduced). All the peak intensities were normalised to peak u4 which remains unaltered in any one preparation. The glucose concentration was also monitored (not shown) and was observed to decrease linearly to about half its initial value over the period shown in fig.4.

The pH externally was measured at the beginning and end of the experiment by a Pye model 290 pH meter with glass electrodes. The internal pH was measured by the histidine C(2) resonances of haemoglobin. The internal pH remained constant at pH 7.41 ± 0.03 and the external remained constant at 7.46 ± 0.05. In this set of data, no significant induction period for the appearance of the lactate peak was detected. The rate of production of lactate seemed to be independent of whether glutathione was being reduced or not. The glutathione reduction was nearly complete after 30 min in agreement with values given previously [18].

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