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F. F. Brown and I. D. Campbell

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N.m.r. studies of red cells

By F. F. Brown and I. D. Campbell
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU

Recent n.m.r. studies of intact red cells are described. With ^{1}H n.m.r. the normal high resolution spectra of red cells, even at high fields, are relatively uninformative because the very large number of resonances from the cells merge into a broad envelope. If a simple 90–τ–180° spin echo pulse sequence is used, however, many resonances can be resolved. These include signals from haemoglobin histidines, glutathione, lactate and pyruvate. ^{13}C and ^{31}P signals have also been seen with a spectrometer converted to observe these nuclei essentially simultaneously.

N.m.r. is well suited to monitor the time course of events after a perturbation of the cell system. Lactate increase, glutathione recovery after oxidation and alkylation of glutathione by iodoacetate can all be observed directly in red cell suspensions by means of ^{1}H spin echo n.m.r. This method has also been used to measure isotope exchange (^{1}H–^{2}H) of lactate and of pyruvate at both the C-3 and the C-2 positions, and some of these exchange rates can be interpreted in terms of the activity of specific enzymes in the cells. ^{1}H spin echo n.m.r. has also been used to obtain information about the transport rates of small molecules into cells.

By means of the $^{13}C/^{31}P$ spectrometer and [^{13}C-1] glucose, the ^{13}C enrichment of 2,3-diphosphoglycerate (2,3-DPG) can be monitored at the same time as the levels of 2,3-DPG, ATP and inorganic phosphate are observed by ^{31}P n.m.r.

Introduction

Over 300 years ago Van Leeuwenhoek (1675) reported the observation of red cells by means of a microscope. Since then the red cell and its contents have continued to be among the first to be studied by any new technique. The picture of the red cell that has emerged from these techniques can be summarized in the schematic diagram shown in figure 1 (for reviews see Rose 1971; Surgenor 1974).

The biconcave disk shape, the elastic properties of the cell, and the ion gradients require that ATP levels be maintained from a glucose supply. The cell and its contents are protected in a variety of ways. For example, glutathione protects against oxidation and, if itself oxidized, is regenerated from NADPH produced in the pentose phosphate pathway (p.p.p.). Another example of regeneration is the reduction of ferric haemoglobin to the ferrous form by an enzyme that can utilize NADH or NADPH. The main component of the cell, haemoglobin, has been exhaustively studied in its purified state and the sophisticated molecular properties of this protein are now well understood (Baldwin 1975). These properties include the ability to change the O_2-binding characteristics in response to changes in pH or the concentration of the metabolite 2,3-diphosphoglycerate (2,3-DPG).

What can n.m.r. add to this wealth of knowledge? The main advantage of the technique is that it allows many molecules to be examined inside intact cells. One goal, therefore, would be to check that conclusions reached from studies of isolated and purified materials are valid when applied to the concentrated and specialized interior of the cell. There are, of course,

technical problems which must be considered; these include low sensitivity and lack of resolving power, as well as the problems of maintaining cells in a viable condition in the n.m.r. instrument. Fortunately, it looks as if many of these can now be at least partially overcome, mainly because of considerable improvements in n.m.r. spectrometers in recent years.

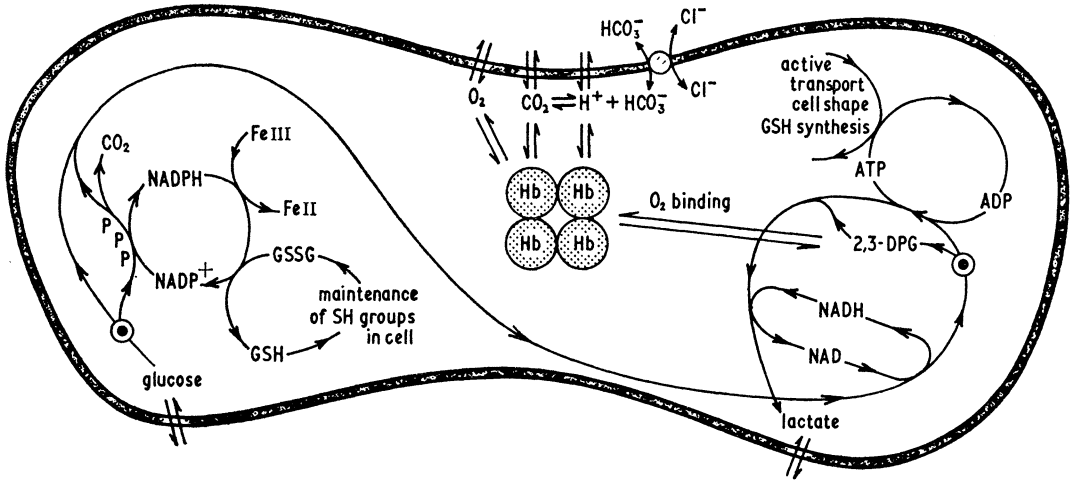

FIGURE 1. Schematic representation of the red cell, showing some of the interactions of the glycolytic pathway that maintain cell contents and shape. GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); Hb, haemoglobin; P, phosphate.

The first n.m.r. study of intact red cells that we know of was by Odeblad *et al.* (1956), who conducted ^1H n.m.r. experiments to measure the exchange rate of ^1H_2O with ^2H_2O in cells. The water in red cell suspensions has continued to be studied, for example to measure membrane permeability (Fabry & Einsenstadt 1978, and references therein) and to investigate the polymerization of sickle cell Hb (Cottam & Waterman 1976; Zipp *et al.* 1977). The ions sodium and bicarbonate have also been studied by ^{23}Na (Yeh *et al.* 1973) and by ^{13}C n.m.r. (Matwiyoff & Needham 1972). In an important paper, Moon & Richards (1973) demonstrated that 2,3-DPG and inorganic phosphate could be observed inside red cells by ^{31}P n.m.r. and that resonances due to these species could be used to measure intracellular pH. Henderson *et al.* (1974) then showed that the response of intracellular ATP and 2,3-DPG to external stimuli could be followed.

In this paper we review some of our recent work on red cells. This work began with the realization that the histidine resonances of haemoglobin could be observed to titrate in suspensions of whole cells (Brown *et al.* 1976) and also that glutathione and the metabolites lactate and pyruvate could be studied (Brown *et al.* 1977) by ^1H n.m.r. Subsequently we have shown that the transport of small molecules into cells can be studied by ^1H spin echo n.m.r. (Brindle *et al.* 1979) and that ^{13}C and ^{31}P signals can be observed essentially simultaneously by means of a modified spectrometer (Styles *et al.* 1979).

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Experimental

Sample preparation. The red cells were usually prepared from freshly drawn venous blood, as described previously (Brindle *et al.* 1979). The haematocrit value, measured on a Hawksley microhaematocrit centrifuge, was adjusted to about 80%. All measurements were made at 37 °C.

^{1}H *n.m.r.* The spectra were obtained with a 270 MHz spectrometer with a BNC 12 computer and Diablo disk drive. A spin echo 90°–τ–180° pulse sequence with τ = 60 ms and overall repetition time of *ca.* 1 s were used. The accumulated transient signals (56–512 scans) were stored on a computer disk by means of an automatic data acquisition routine.

^{13}C–^{31}P *n.m.r.* A wide bore magnet operating at 4.2 T was used with a solenoidal probe, double tuned to ^{13}C and ^{31}P frequencies. The ^{13}C and ^{31}P signals were collected during alternate scans, with a total collection time of about 20 min. Automatic collection routines were again used (Styles *et al.* 1979).

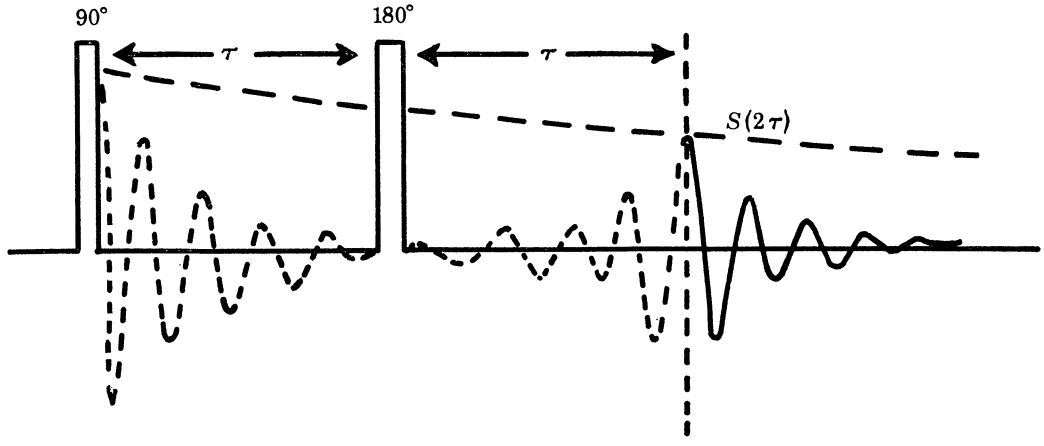

Figure 2. Spin echoes. Illustration of the refocusing of the magnetization in an n.m.r. experiment by a 180° pulse after a given time. $S(2\tau)$ is given by equation (1).

Methods

^{1}H *n.m.r.*

This is more sensitive than ^{31}P or ^{13}C n.m.r., but has the disadvantage that very many resonances occur in a narrow range, thus making the resolution problem severe (Campbell & Dobson 1979). Because of this problem little ^{1}H work, other than the observation of the water resonance, has been carried out on cells.

All the ^{1}H n.m.r. work described here was carried out by means of spin echo methods. A simple two pulse sequence 90°–τ–180° produces an echo at time 2τ after the 90° pulse (Carr & Purcell 1954); see figure 2. The amplitude of the echo is given approximately by

$$S(2\tau) = S(0) \exp(-2\tau/T_2 - 2D\gamma^2G^2\tau^3/3) F(J), \quad (1)$$

where γ is the magnetogyric ratio, G is the magnetic field gradient experienced by a molecule diffusing with coefficient D and $S(0)$ is the echo amplitude after the 90° pulse. The $F(J)$ term leads to a modulation of the signal if there is homonuclear spin–spin coupling in the system. For a singlet, $F(J) = 1$ at all values of τ, but for a first order doublet, $F(J)$ is of the form $\cos(2\pi J\tau)$ (Freeman & Hill 1975). (For a more precise equation for $S(2\tau)$, see Packer 1973.)

$S(2\tau)$ can thus be considered to be a function of three variables: (*a*) the intrinsic T_2 of the observed nuclear resonance, a parameter that depends on the mobility of the molecule observed; (*b*) the spin–spin coupling patterns of the resonances and (*c*) a term arising if the molecule diffuses to a region of different applied magnetic field during the refocusing period 2τ. Each of these dependencies is, as we shall show, of value in studies of whole cells. A useful feature of the spin echo methods is that the dependencies on J and G^2D can be removed by using a train of closely spaced 180° pulses to refocus the magnetization, rather than a single pulse (Freeman & Hill 1975; Campbell & Dobson 1979).

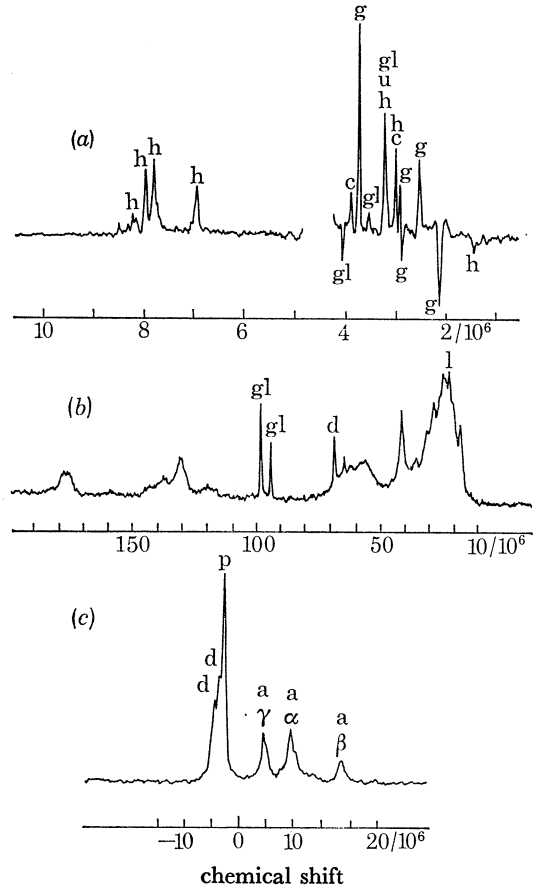

FIGURE 3. N.m.r. spectra from red cells: (*a*) ^{1}H spin echo spectrum shows haemoglobin (h), glucose (gl), glutathione (g), creatine (c), and unassigned (u) peaks. The magnetic field was 6.3 T, the sample volume, 0.5 ml, and the accumulation time, 4 min. The medium was ^2H_2O Krebs buffer with 10 mM glucose. (*b*) ^{13}C spectrum shows peaks from labelled lactate C-3 (l), glucose C-1 (gl) and 2,3-diphosphoglycerate C-3 (d). The field was 4.3 T, the sample volume, 3.5 ml, and the accumulation time, 10 min. The medium was ^1H_2O Krebs buffer with 10 mM glucose enriched at the 1-position. (*c*) ^{31}P spectrum shows peaks from 2,3-diphosphoglycerate (d), inorganic phosphate (p) and ATP (a). Conditions were the same as for spectrum (*b*).

Perhaps the most important aspect of the spin echo technique is its ability to simplify spectra (Campbell *et al.* 1975). For example, $S(2\tau)$ is small for molecules with short T_2 values and thus these components can be eliminated from a spectrum by choosing a suitable τ value. In red cells, for example, a τ value of 60 ms yields a relatively well resolved and informative spectrum, as shown in figure 3*a*. (Brown *et al.* 1977).

It may be observed in figure 3*a* that there is inversion of some peaks. This is due to the $F(J)$ term in equation (1). The modulation behaviour of resonances is a useful assignment

aid when comparisons are made with spectra of model compounds. Another advantage of the $F(J)$ term is that the properties of an unresolved resonance may be detected in an observed resonance to which it is spin coupled. An example of this is given by the proton resonances of lactate. These consist of a single proton (the C-2 proton) quartet at about $4/10^6$ and a three proton (C-3 methyl) doublet at about $1.3/10^6$. If the C-2 proton is exchanged for a deuteron the homonuclear coupling between these resonances is removed and at 60 ms, for example, $F(J)$ changes from approximately -1 to $+1$. $F(J)$ can also be changed by double resonance (Campbell & Dobson 1975, 1979); see isotope exchange section below.

The term involving G^2D in equation (1) is important if the observed molecule diffuses to a region of different field during the period 2τ. We have shown that this term can produce significant damping of echo amplitude in suspensions of red cells if there is a difference in magnetic susceptibility between the inside and the outside of the cells (Brindle *et al.* 1979). This is especially true in regions outside vesicles and cells. Since this is so, the echo intensity from molecules in different compartments can be different, and this can be used to measure the transport of small molecules into cells. The addition of an inert paramagnetic complex that is not transported enhances these effects (see below).

^{31}P *and* ^{13}C *n.m.r.*

^{31}P n.m.r. has been used extensively in studies of intact tissue and ^{13}C n.m.r. is being used increasingly. The information obtained with these nuclei is complementary. Thus ^{31}P that is 100% abundant can be used to observe the concentrations of intracellular metabolites and intracellular pH while ^{13}C, whose natural abundance is low, can be used to determine the fate of an enriched metabolite. These nuclei also have some technical features in common, for example, both require irradiation of coupled ^{1}H nuclei to simplify the spectra. In view of these considerations, and to avoid problems of variability in behaviour between cell preparations, a probe was constructed that allowed ^{13}C and ^{31}P n.m.r. signals to be collected, from a single sample, during alternate scans (Styles *et al.* 1979).

Time courses

Although it is possible to use n.m.r. to determine absolute concentrations of small molecules in cell suspensions, there are some difficulties involved. Relative concentrations, however, can be determined more readily and n.m.r. is well suited to follow the time course of events after a perturbation to a cell system. In this paper we will give several examples of time courses, including lactate growth, isotope exchange, fate of enriched glucose, and membrane transport.

To facilitate the measurement of such time courses we have developed computer programs that, after the initial perturbation, automatically collect data, storing batches of information at known intervals and stepping the time scale to make it suitable for an exponential variation in peak intensity with time.

Results and discussion

Many of the molecules that can be observed in n.m.r. studies of red cells are indicated in figure 3. Lactate and pyruvate as products of glycolysis appear in the ^{1}H spectra, and small molecules such as amino acids can be observed if added to the cells (Brown *et al.* 1977; Brindle *et al.* 1979). Some of the experiments carried out on these various molecules are described below.

Haemoglobin

In ^1H spectra of red cells several histidines can be observed to titrate as the external pH is varied. Comparison of these titrations with those for purified haemoglobin (Brown & Campbell 1976) can be used to measure the internal pH (Brown *et al.* 1977). The spectra are also sensitive to the state of oxygenation of the haemoglobin, but these effects have not yet been properly studied.

Glutathione

Human red cells contain about 3 mM reduced glutathione. The ^1H spin echo spectra of the protons on the α-carbon of the glycine (at chemical shift $3.76/10^6$) and the S-linked carbon of the cysteine residue (at $2.9/10^6$) are sensitive to the oxidation state of the molecule. This has been used to measure the metabolic flux through the pentose phosphate pathway by means of the rate of regeneration of reduced glutathione after oxidation by *t*-butyl hydroperoxide (Brown *et al.* 1977). The alkylation of the free SH group by iodoacetate can be followed by observing the appearance of the resonance of the *S*-carboxymethyl derivative at $3.2/10^6$. We have used this to show that the alkylation rate at pH 7.4 is the same inside cells as it is in free solution.

Lactate production

Lactate production in cell suspensions is readily followed by a spectroscopic enzyme assay with lactate dehydrogenase and NAD^+, especially in red cells, where lactate transport is rapid (Dubinsky & Racker 1978). In some circumstances, however, it is convenient to measure this also by n.m.r. because of the rapidity with which measurements can be made and the ability to monitor other parameters at the same time. There is also no problem of interference from haemoglobin absorption bands, c.f. the spectroscopic assay.

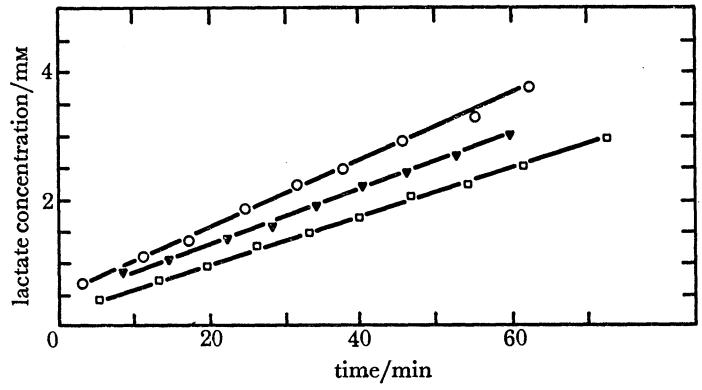

FIGURE 4. Lactate production as monitored by the methyl resonance at $1.3/10^6$ in a red cell suspension. The red cells were in H_2O Krebs Ringer buffer and the strong H_2O peak was suppressed with a long pulse (Campbell & Dobson 1979). The medium contained (○) 20 mM dihydroxyacetone + 10 mM glucose, (▼) 20 mM dihydroxyacetone alone and (□) 10 mM glucose alone.

Figure 4 shows the increase in lactate, as measured by ^1H n.m.r., as a function of time. The spectra were obtained from cells in 100% ^1H_2O Krebs/Ringer buffer with a capillary of ^2H_2O for a field lock. A long, selective pulse was applied to the sample to suppress the ^1H_2O resonance (Brown *et al.* 1977; Campbell & Dobson 1979). Dihydroxyacetone has been shown to be utilized by red cells after it has been phosphorylated by a triokinase to dihydroxyacetone phosphate (Beutler & Guinto 1973). This then enters glycolysis via triose phosphate

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isomerase. It may be observed that lactate increase from dihydroxyacetone plus glucose is faster than from glucose or dihydroxyacetone alone. The relative rates for metabolism of glucose and dihydroxyacetone were found to vary from person to person and with time of day for a given person.

The 2,3-DPG *bypass*

An important feature of red cell metabolism is the bypass around the step catalysed by phosphoglycerate kinase; instead of conversion of 1,3-DPG to 3-phosphoglycerate with the generation of an ATP molecule from ADP, 1,3-DPG is converted by a mutase to 2,3-DPG, which is then degraded by a phosphatase to phosphoglycerate (3-PG) (Duhm & Gerlach 1974). The level of 2,3-DPG in human red cells is high, around 5 mM, but this concentration varies considerably depending on conditions, e.g., the blood storage, oxygen deficiency and pH (Duhm & Gerlach 1974). Two roles for the bypass have been suggested (Rapoport 1968; see also Chiba & Sasaki 1978): (*a*) to generate 2,3-DPG as an allosteric modifier of the O_2-binding affinity of haemoglobin, and (*b*) as an idling cycle, so that glycolysis can be maintained without generating ATP.

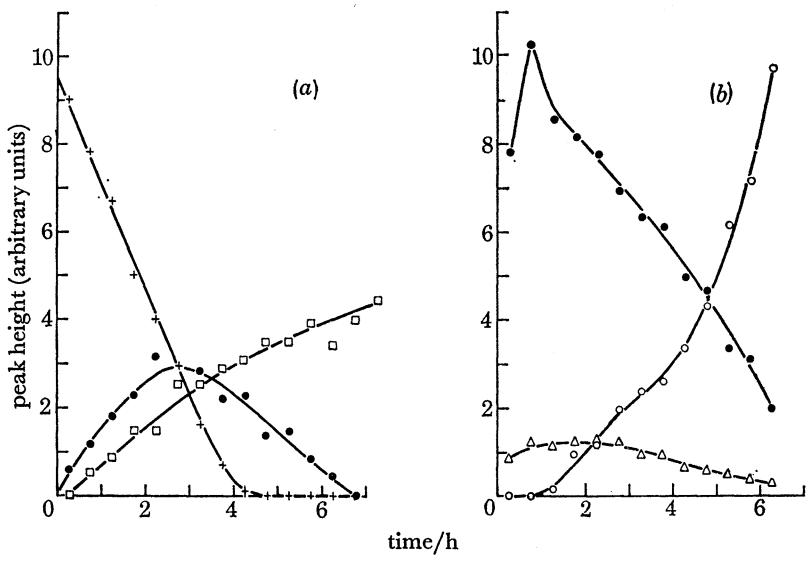

FIGURE 5. Simultaneous ^{13}C, ^{31}P time courses of red cell metabolism. The ^{13}C graphs (*a*) show the change in the concentration of labelled glucose C-1 (+), lactate C-3 (□), and 2,3-DPG C-3 (●). The ^{31}P graphs (*b*) show the change in the total pools of 2,3-DPG (●), inorganic phosphate (○) and ATP (△). The ATP was monitored through its β peak, which is not overlapped by any other peaks. The conditions were those used to obtain figures 3*b* and 3*c*.

As illustrated in figure 5, the simultaneous collection of ^{13}C and ^{31}P data can give interesting information about the bypass. In this experiment, 10 mM glucose, enriched at the 1-position, was added to a suspension of washed, freshly drawn cells. The utilization of glucose, the incorporation of label into 2,3-DPG and the subsequent loss of label from this molecule to lactate are all observable in the ^{13}C time course. In the ^{13}P time course the decay of 2,3-DPG and of ATP concentrations and the increase of inorganic phosphate can be observed. Thus, in this sort of experiment information is obtained about the phosphatase and mutase activities in the bypass, as well as the levels of some of the metabolites. The 1,3-DPG and 3-PG concentrations are too low to observe directly.

Isotope exchange of lactate and of pyruvate

If the solvent is ^2H_2O, the protons attached to the C-2 and C-3 carbons of lactate and pyruvate can be lost to solvent (Rose & Rose 1969; Saur *et al.* 1968). This occurs both for endogenous lactate derived from glucose by metabolism and for pyruvate or lactate added to a suspension of cells in ^2H_2O.

The endogenous lactate, under the conditions used in our experiments, has a methyl resonance that was at lower concentration than that calculated from assay of lactate by standard enzyme methods. This is to be expected, since deuterium is incorporated at the pyruvate kinase step (Robinson & Rose 1972) and probably at the phosphoglucose isomerase step.

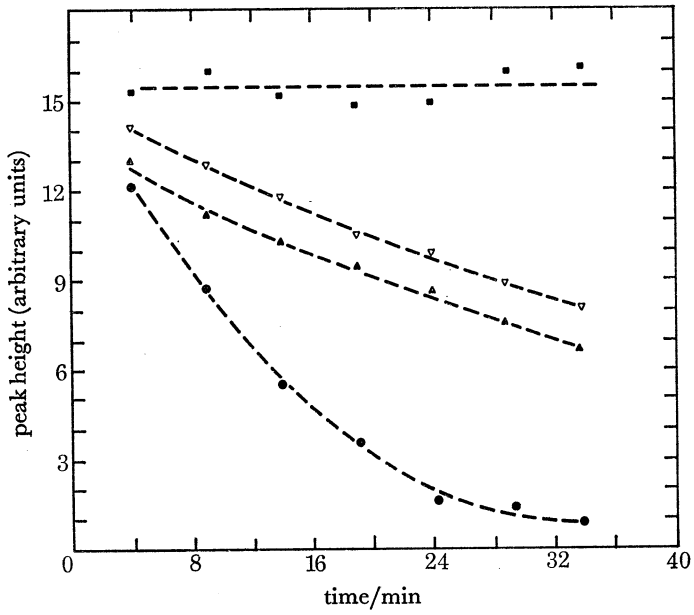

FIGURE 6. Proton C-3 exchange in red cells. The graphs show the change in the proton content of the lactate methyl in lysed cells (■) and in whole cells (▽) and of the pyruvate methyl (at 2.36/10⁶) in lysed cells (●) and in whole cells (△); 10 mM lactate and 10 mM pyruvate were added to the sample at time $t = 0$.

If lactate and pyruvate are added to a cell suspension, then molecules are transported into the cells and acted on by the enzymes in the cell; C-2 exchange has already been noted in red cells (Rose & Warms 1969).

Figure 6 shows typical results for C-3 exchange when 10 mM lactate and 10 mM pyruvate are added to red cells. The lactate and pyruvate methyl resonances disappear at equal rates. To avoid complications arising from C-2 exchange occurring at the same time as the C-3 exchange, the C-2 resonance was irradiated during the refocusing time, 2τ (see below). We have not yet ascertained the cause of C-3 exchange in red cells, but our current evidence does not support the view that pyruvate kinase or glutamic pyruvic transaminase are important as has been suggested for other systems (Rognstadt & Wals 1976; Walter *et al.* 1975).

While the cause of C-3 proton exchange is unknown, the exchange at C-3 allows the equilibrium rate of the methyls of lactate and pyruvate across lactate dehydrogenase to be measured. Thus, in whole cells the methyls are in relatively rapid exchange and the lactate

and pyruvate peaks disappear at the same rate. In lysates, however, while pyruvate is deuterated more rapidly than in whole cells, the lactate deuteration rate is slow. This implies that equilibrium across lactate dehydrogenase is relatively slow in lysates. The reason for this is still uncertain, but it is tempting to propose some sort of compartmentation, which is destroyed by lysis. [*Note added in proof.* An important factor is extracellular NADase activity.]

Exchange at the C-2 lactate position is more complex than C-3 exchange or methyl equilibration. The enzymes glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase are coupled to the coenzyme NADH, which stereospecifically transfers hydrogen from lactate to glyceraldehyde phosphate. This proton is then lost to solvent at the steps catalysed by the enzymes aldolase and triose phosphate isomerase (Rose & Rose 1969), as illustrated in figure 7.

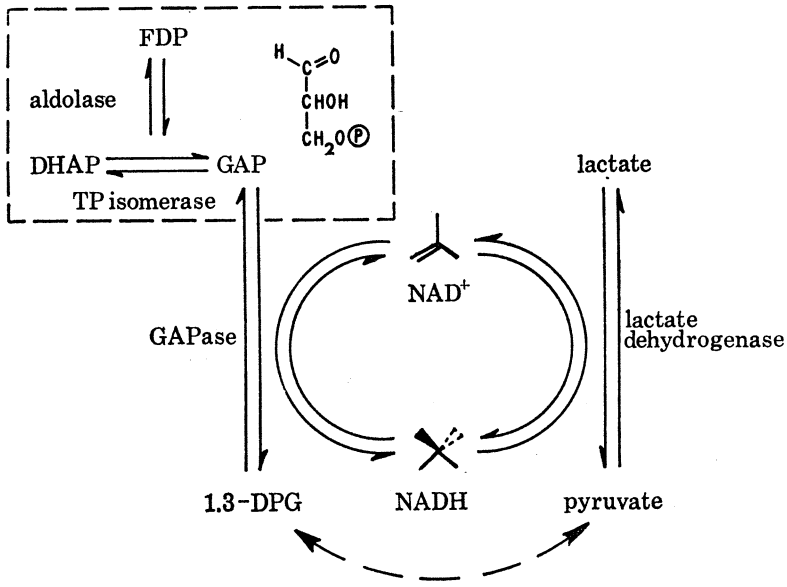

FIGURE 7. Mechanism of proton C-2 exchange in red cells. This pathway illustrates the stereospecific transfer of protons from lactate C-2 via NAD/NADH to glyceraldehyde phosphate (GAP) followed by transfer to dihydroxyacetone phosphate (DHAP) and thence to solvent via the aldolase equilibrium. DPG, diphosphoglycerate; FDP, fructose diphosphate, TP, triose phosphate.

A typical C-2 exchange measurement is shown in figure 8, for which 6 mM exogenous lactate was added to the cells at $t = 0$. The loss of the C-2 proton due to exchange removes the homonuclear coupling to the methyl resonance, so that the methyl resonance changes from a negative-going peak to a positive-going one because of the $F(J)$ term in equation (1). The half life for the exchange in figure 8 is about 7 min, a typical result for fresh cells. When the C-2 exchange rate is slower, significant C-3 exchange may occur during the experiment. Correction for C-3 exchange can be made by carrying out interleaved decoupling of the C-2 resonance. There is also a small isotope shift of the methyl resonance when a deuteron replaces a proton.

We have found C-2 exchange to be very sensitive to the metabolic state of the cells. This exchange is inhibited by iodoacetate, lysis and pyruvate. We have also noted distinct differences between the C-2 exchange rates of normal blood and of blood from patients with a pyruvate kinase deficiency.

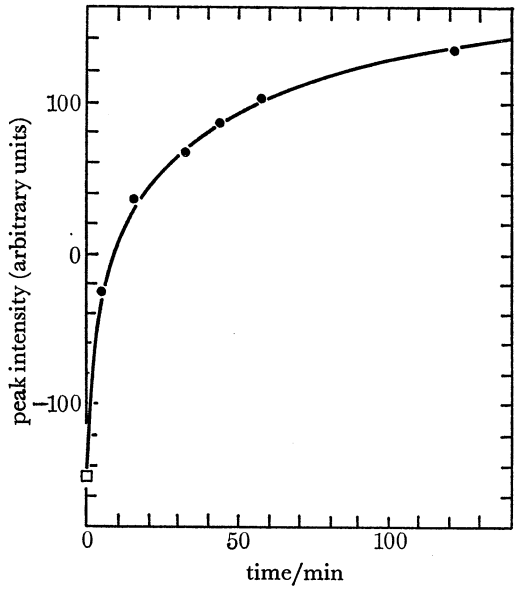

FIGURE 8. Exchange of lactate C-2 proton in red cells; 6 mM lactate was added at time $t = 0$. Under these conditions, where the C-3 exchange rate is relatively slow, the change from the inverted to the non-inverted signal follows the change from homonuclear to heteronuclear coupling as the C-2 hydrogen is replaced by deuterium.

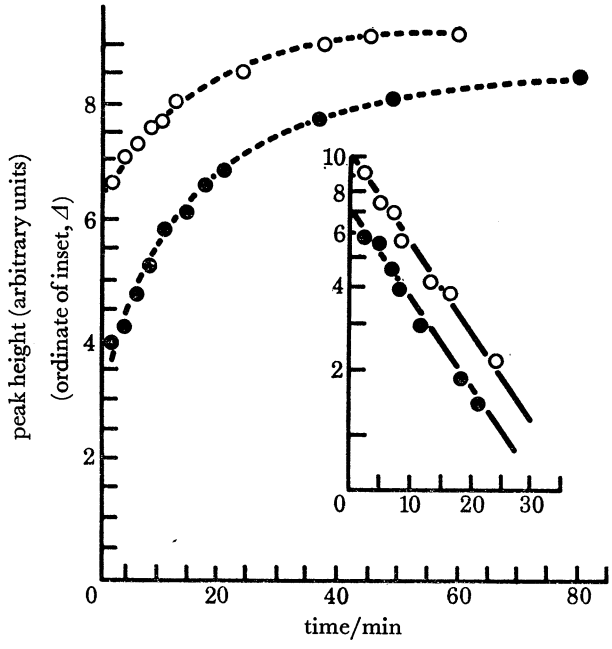

FIGURE 9. Alanine influx into red cells. 6 mM Alanine was added to a red cell suspension in the absence (○) and in the presence (●) of DyDTPA to enhance the differential magnetic susceptibility. Inset: semi-logarithmic plot of Δ against time, where $\Delta = [I_\infty - I(t)]/I_\infty$ and I is the height of the alanine methyl resonance.

Membrane permeability

In studies of whole cells, it is desirable to have the ability to measure transport of metabolites between compartments as well as to measure the concentrations and fluxes of metabolites as described above. We have shown that ^{1}H spin echo n.m.r. can be used to measure the influx of small molecules into red cells (Brindle *et al.* 1979).

The method depends on the molecules on the two sides of the cell membrane giving rise to different intensities in the n.m.r. spectrum. The difference in intensities allows changes in the distribution of molecules to be measured as a time course. The intensity differences arise because magnetic susceptibility differences ($\Delta\chi$) between the inside and outside compartments generate local field inhomogeneities. For spheres the inhomogeneities arise only outside the spheres, but, in general, the inhomogeneities inside and outside depend on the cell shape. The field gradients or inhomogeneities are directly proportional to $\Delta\chi$, which can be increased by the addition of a paramagnetic metal ion surrounded by inert ligands. We have found the complex dysprosiumdiethylene–triaminepentaacetic acid (DyDTPA) to be suitable for this and concentrations of this complex of less than 1 mM to be sufficient.

When $\Delta\chi$ is large enough, the field gradients produced cause some line broadening, but a more important effect is that the echo is damped due to the G^2D term in equation (1). It turns out that the distance diffused by small molecules in the time $2\tau = 120$ ms is of the same order as the cell dimensions, hence the importance of G^2D term.

Figure 9 shows results of an experiment on the influx of alanine into red cells. The height of the alanine methyl resonance, with and without the addition of DyDTPA, is plotted as a function of time. It may be noted that the complex has no effect on the observed influx rate, as shown in the log plot.

Conclusions

We have shown that ^{1}H spin echo n.m.r. is a powerful addition to the tools available for studying cells. It can be used to examine haemoglobin and glutathione inside cells and to investigate the 2,3-DPG bypass. Lactate growth and isotope exchange of lactate and of pyruvate are also readily studied, as is the transport of small molecules into the cells.

Given these new methods, it remains to apply them over the next few years in a way that produces new biochemical information. There are already indications that the unique ability of the n.m.r. method to examine the properties of enzymes inside intact cells is of considerable value; significant differences between *in vivo* and *in vitro* properties are beginning to emerge.

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