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Application of Spin-Echo Nuclear Magnetic Resonance to Whole-Cell Systems

MEMBRANE TRANSPORT

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A new method for studying membrane transport is presented. High resolution n.m.r. is used to measure the distribution of small molecules between the intracellular and extracellular compartments. The method uses spin-echo techniques and relies on a difference in the magnetic susceptibility of the media inside and outside of cells. It also provides simultaneous information on the metabolic status of the cell. The method is illustrated by a study of alanine and lactate transport in the human erythrocyte.

N.m.r. is being used increasingly to study whole-cell suspensions, especially by ^{31}P n.m.r. (Moon & Richards, 1973; Navon *et al.*, 1978). Such studies have shown that various metabolite concentrations and intracellular pH can be measured. Detailed metabolic information is also available from n.m.r. studies of the more sensitive and ubiquitous ^{1}H nucleus (Brown *et al.*, 1977), but such measurements rely on the spectral simplification achieved by applying spin-echo techniques (Campbell *et al.*, 1975). In an extension of our previous ^{1}H spin-echo n.m.r. studies on whole cells, we demonstrate here a means of applying this technique so as to observe directly the membrane transport of any molecule that gives an observable n.m.r. signal. This information on transport can be obtained at the same time as the other information on the cell previously described.

Studies of transport involve the determination of the distribution of the molecule of interest across the cell membrane as a function of time. Continuous monitoring procedures include: (*a*) light scattering to measure volume changes caused by the osmotic response to the changes in molecular distribution (Sen & Widdas, 1962; Sha'afi *et al.*, 1967); (*b*) enzymic modification of specific extracellular species (Hertz & Barenholz, 1973). Separation procedures involve filtration or centrifugation and the determination of the distribution of a radioactively labelled molecule. Chemical methods are often necessary for stopping transport at exact time intervals (Eilam & Stein, 1974).

N.m.r. studies of transport in cell suspensions depend on some method for distinguishing molecules inside and outside the cells. Transport of carboxylic acids into membrane vesicles has been studied by exploiting chemical-shift differences caused by pH gradients across the membrane (Cramer & Prestegard, 1977). Differential relaxation on the two sides of the membrane has also been used to measure membrane permeability. For example, water transport into erythrocytes has been measured by ^{17}O n.m.r. (Shporer & Civan, 1975) and by ^{1}H n.m.r. by adding Mn^{2+} ions to the outside medium (Conlon & Outhred, 1972). Lanthanide ions have also been used in vesicle systems to cause rapid relaxation of the resonances of molecules outside the membrane (Hunt, 1975; Degani, 1978). Enzyme-catalysed exchange of ^{1}H for ^{2}H in a ^{1}H n.m.r. experiment has been used to observe the efflux of L-alanine from erythrocytes (Brindle, 1978); the ^{2}H labelling of alanine was achieved by addition of glutamate–pyruvate transaminase (EC 2.6.1.2) to the extracellular space. These methods are, however, difficult to apply to a wide variety of molecules and therefore lack generality.

In this paper we present an n.m.r. method which does appear to have the potential for widespread application. The transport of any molecule with an observable n.m.r. signal can be measured relatively easily. 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. This difference in signal intensities allows changes in the distribution of molecules to be measured as a function of time. The intensity differences arise because of magnetic susceptibility effects and from the use of spin-echo techniques.

Experimental

The erythrocytes from various subjects were prepared at room temperature from freshly drawn venous blood by washing once in 0.9% NaCl in $^{1}H_2O$ and either twice in $^{1}H_2O$ Krebs/Ringer buffer or four times in $^{2}H_2O$ Krebs/Ringer buffer (Krebs &

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Henseleit, 1932) that was thoroughly gassed with O_2/CO_2 (19:1). All solutions contained 10mM-glucose. The samples were run on a Bruker 270 MHz FT n.m.r. spectrometer fitted with quatrature detection. Spectra were accumulated in the spin-echo mode with a 90°–τ–180°–τ pulse sequence (90° pulse 15μs) where τ is the delay time, usually 60ms and with 1s overall repetition rate as previously described (Brown *et al.*, 1977). The accumulated decays (10–512 scans) were stored on disc by a computer-controlled automatic data acquisition routine, which also provided accurate timing for all rates of transport studied.

On completion of an experiment, the haematocrit of the sample was measured on a Hawkesley micro-haematocrit centrifuge.

The addition of substrate species to the erythrocyte suspensions was made from iso-osmotic solutions. The samples were in 5mm diameter tubes containing 0.5ml of suspension. Unless otherwise stated, all samples were run at 37°C and preheated in a water bath before mixing. For the more rapidly transported species, it was found that preheating the sample was sufficient to permit temperature equilibration within 15s and measurement could be started within 30s.

To prevent sedimentation in low haematocrit samples, air bubbles were passed through the sample tube between data accumulations. The air was administered through a fine plastic tube from a peristaltic pump and the bubble rate monitored by the temporary shift in the lock signal each time a bubble passed through the receiving coil. This was satisfactory for haematocrits below 70%, but was not suitable or necessary for very viscous high-haematocrit samples.

The compounds used to enhance the differential magnetic susceptibility included Cationised Ferritin (Miles Laboratories, Stoke Poges, Slough, U.K.) and AnalaR chlorides of Fe^{3+}, Mn^{2+} and Dy^{3+} (BDH Chemicals, Poole, Dorset, U.K.). These metal ions were liganded to desferrioxamine (CIBA Laboratories, Horsham, West Sussex, U.K.), EDTA or 'diethylenetriamine penta-acetic acid' (*NN*-bis-{2-[bis(carboxymethyl)amino]ethyl}glycine, DTPA) (BDH). D- and L-Alanine and L-lactate were obtained from BDH.

Physical Principles of the Methods Used

Spin-echoes

The n.m.r. spectra in this paper are obtained using spin-echo methods followed by Fourier transformation. The collection of spectra using a two-pulse spin-echo sequence has the advantage that resonances with relatively long values of T_2 can be selected from a spectrum (Campbell *et al.*, 1975; Brown *et al.*, 1977). The simple two-pulse sequence 90°–τ–180° produces an 'echo' at time 2τ after the 90° pulse (Carr & Purcell, 1954), with amplitude given by:

$$S(2\tau) = S(0)\exp\left\{-\frac{2\tau}{T_2} - \frac{2D\gamma^2 G^2 \tau^3}{3}\right\} F(J) \quad (1)$$

where γ is the magnetogyric ratio, G is the magnetic field gradient across the sample and D is the diffusion coefficient of the observed molecule in the solution. 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 times, but for a first-order doublet $F(J) = \cos(2\pi J\tau)$ (Freeman & Hill, 1975). This makes it a useful assignment aid. For example, the alanine methyl resonance is coupled to the α-CH with coupling constant $J = 7.3$ Hz, thus when $2\tau = 136$ms the methyl resonance is inverted because $F(J) = -1$.

The physical meaning of the term involving τ^3, G^2 and D is that the echo is not properly refocused if a molecule diffuses to a region of different applied field during the time required to produce the echo. In a spectrometer with good homogeneity and with a homogeneous sample, this term is negligible for τ values of up to at least 100ms. However, at long values of τ, or when G is large, this term makes the amplitude of the echo decay rapidly (Abragam, 1961) and with calibrated applied field gradients this is a good method for measuring diffusion coefficients (Stejskal & Tanner, 1965). Andrasko (1976) has measured Li^+ transport into erythrocytes using a method that utilizes the fact that the diffusion of molecules inside a cell is more restricted than outside. Pulsed field gradients were applied to the sample in a way which allowed the fraction of Li^+ ions inside the cell to be measured.

The effects of the G^2D term can be removed by applying many 180° pulses between the 90° pulse and data collection since τ is then kept short as far as this term is concerned. A suitable pulse sequence is the Carr–Purcell–Meiboom–Gill sequence with $\tau \simeq 1$ ms (Meiboom & Gill, 1958; Freeman & Hill, 1975). With this sequence the decay rate is dominated by the T_2 term.

Fig. 1 demonstrates the behaviour of the echo amplitude for extracellular glycine as a function of time in three different situations. In ^2H_2O Krebs/Ringer buffer the amplitude of the α-CH_2 resonance decays at a rate that corresponds to a half-life of 1.5s with both the simple two-pulse sequence and the multiple-pulse sequence. In an 80% suspension of erythrocytes, however, the observed decay rate is much faster with the simple sequence (half-life 25ms) than with the multiple-pulse sequence (half-life 280ms). In this experiment all the glycine is effectively outside the cell, because the transport rate is slow and the experiments were done relatively quickly after the addition of glycine. Glycine is also convenient because the α-CH_2 resonance is a singlet and $F(J) = 1$

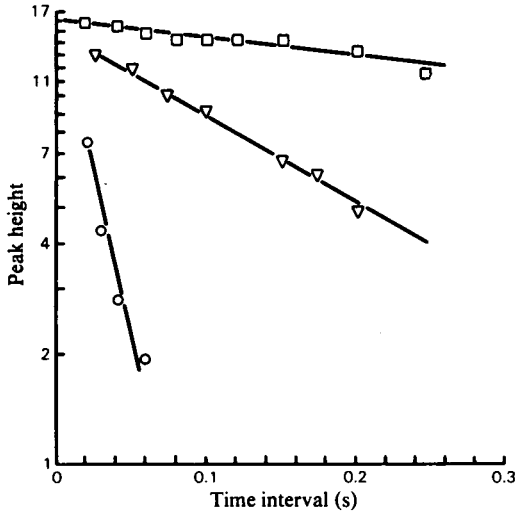

Fig. 1. *Effect of diffusion on spin-echo amplitude*
The height of the CH_2 resonance of glycine observed in spin-echo experiments as a function of the time interval between the 90° pulse and the start of data acquisition. The experiment is arranged so that essentially all the glycine is outside the erythrocyte. Other conditions were: temperature, 293 K; haematocrit, 84%; $[glycine]_{out}$, 75 mM. ○, Simple 90°–τ–180°–τ sequence on the suspension of cells ($[Dy\text{–}DTPA]_{out}$ 0.15 mM); ▽, multiple-pulse sequence (Carr–Purcell–Meiboom–Gill) on the same sample; □, a control experiment on a cell-free solution of glycine (12 mM) and Dy–DTPA (0.15 mM) using a 90°–τ–180°–τ sequence.

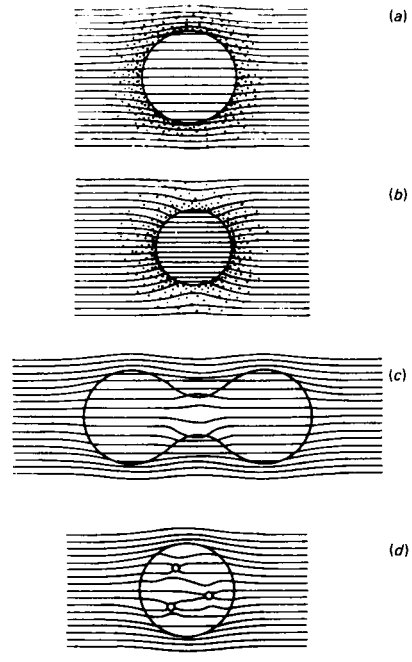

Fig. 2. *Magnetic field in particle suspensions*
Illustration of the effects of different magnetic susceptibility on the lines of magnetic flux in different geometries: (*a*) $\chi_{in} < \chi_{out}$; (*b*) $\chi_{out} < \chi_{in}$; (*c*) representations of lines of flux in the erythrocyte; (*d*) representation of field within cell containing vesicles of different susceptibilities.

in eqn. (1). A dysprosium complex has also been added to the solutions (see below), but the ratio of glycine to this complex has been kept constant by making allowance for the volume taken up by the cells. These results suggest that, if the simple two-pulse sequence is used, the term involving τ^3 dominates the decay rate for molecules in the extracellular space. This, as we will show, is due to field gradients in the extracellular space.

Origin of field gradients

In a suspension of particles, magnetic-susceptibility differences between the particles and the suspending medium can give rise to large field gradients. In the case of spheres these gradients arise only outside the spheres (see Fig. 2). According to Glasel & Lee (1974), who performed experiments with glass beads, these gradients can be represented by the approximate equation:

$$\bar{G} = K(B_0) \cdot \Delta\chi \cdot r_0^2/[(r_0^2 + r_0 r + r^2)r] \qquad (2)$$

where K is a constant, B_0 is the applied magnetic field, $\Delta\chi$ is the difference in magnetic susceptibility between inside and outside media, r_0 is the radius of the sphere and r is the distance from the centre of the sphere to the point at which the mean gradient $\bar{G}$ is measured ($r > r_0$). Just outside the surface of the sphere $\bar{G}$ is proportional to $1/r_0$, but this falls off as r increases. It is convenient to define a region in which the field gradients are significant, the shaded area in Fig. 2.

The magnitude of the field gradients and the extent of the shaded area are obviously sensitive to cell shape. The erythrocyte departs significantly from a sphere and some gradients will be produced inside as well as outside the cell as shown in Fig. 2(*c*). In general, however, a cell will have more concave than convex surfaces on the inside and the field gradients will be greater in the external medium. This would not apply, however, if the cell contained small vesicles or particles of different magnetic susceptibility since large gradients could then be produced in the cytoplasm (Fig. 2*d*).

Since the field gradients depend on $\Delta\chi$, they can be controlled by increasing either intracellular or extracellular paramagnetism. Since deoxy- and methaemoglobin are paramagnetic, it is easy to change $\Delta\chi$ from within the erythrocyte. Alternatively, a

paramagnetic species can be added to the outside medium provided that it is not transported.

The added complex increases G in eqn. (1) by increasing $\Delta\chi$ in eqn. (2). Since it is only the bulk susceptibility of each medium which is involved, it is not necessary for the paramagnetic species, e.g. a metal ion, to bind to the molecule of interest, and it is convenient to surround the metal ion with inert ligands which help to prevent both its transport and perturbation of the cell physiology.

An increase in $\Delta\chi$ can be achieved by a variety of agents. There are three basic requirements for any compound used to enhance the differential magnetic susceptibility: it should be highly paramagnetic in order to be effective at very low concentrations; it should not cross the membrane to ensure that the susceptibility effects are differential; it should have no effect on the properties of the cell in general and those being studied in particular.

In the experiment described here, many compounds were found to be satisfactory, including Mn^{2+} either alone or as an EDTA complex, ferritin, and the complex between Fe^{3+} and desferrioxamine. The most satisfactory compound tried was Dy-DTPA. This highly paramagnetic, stable, anionic complex produced a marked effect on $\Delta\chi$ at submillimolar concentration.

The fact that the gradients are dominated by the cell dimension is important in the reduction of the echo amplitude. As demonstrated by the data in Fig. 1, field gradients from the magnet in the absence of cells do not prevent complete refocusing of the echo, because they vary only slowly during the diffusion of the molecule. The mean distance diffused by a small molecule in 1 s is a few tens of micrometres. Since the average cell dimension is only around 5 μm, the molecule is very likely to diffuse into a region of different field during the time 2τ when in the vicinity of a cell. If the cell dimensions are much less than the diffusion distance, these effects are reduced (Packer, 1973).

Observed spectrum as a function of time

The peak height of a resonance in a spectrum, R, depends on the amplitude of the echo at time 2τ and on the linewidth of the resonance. Thus if molecules are in an inhomogeneous field, the resulting value of R from a spin-echo spectrum may be reduced because of diffusion effects and because the spread of field values broadens the line. The movement of molecules within the inside and outside compartments is fast on the n.m.r. timescale, whereas it is slow between compartments. Hence an observed n.m.r. line consists of one outside signal plus one inside signal. The net result of these effects is that R varies as molecules redistribute themselves between compartments of different homogeneity.

Consider now the situation when there is a non-equilibrium distribution of molecules in a cell suspension at $t = 0$. The movement of molecules towards equilibrium can be followed, since the observed peak height as a function of time can be represented by:

$$R(t) = n_{out}(t) f_{out} + n_{in}(t) f_{in} \quad (3)$$

where n refers to the number of molecules and the f factors are normalization constants which relate the number of molecules present in the sample to the observed peak height. The f factors are sensitive to a variety of parameters; f_{out} depends on cell density because the field gradients are localized around the cell surface; f_{out} and f_{in} depend on $\Delta\chi$, cell shape, cell dimensions and on the value of T_2 inside and outside the cell.

It is convenient to define a parameter:

$$\Delta(0) = \frac{R(\infty) - R(0)}{R(\infty)} \quad (4)$$

where $R(0)$ and $R(\infty)$ are the observed peak heights at $t = 0$ and after an equilibrium distribution of molecules has been achieved respectively.

Some of the individual contributions to the f factors can be assessed from the values of $\Delta(0)$ obtained under various experimental conditions. In an 80% suspension of erythrocytes, it was observed that for alanine transport in a multiple-pulse experiment, with 120 ms between the 90° pulse and the final echo, that $\Delta(0) = 0.2$. This gave the contributions from the differential T_2 and the extracellular line broadening from the field differences. When the experiment was repeated using a two-pulse experiment when $2\tau = 120$ ms, then $\Delta(0) = 0.65$. This now includes the contribution from the diffusion term. This contribution may be increased by increasing τ. When $2\tau = 240$ ms, it was found that $\Delta(0) = 0.95$.

$\Delta(0)$ also depends on cell density, since the density of high-field-gradient regions in the extracellular space varies directly with cell density. $\Delta(0)$ also depends on the amount of redistribution which takes place during the experiment. In influx experiments, this will increase as the cell density increases, making them more effective at high cell density. In an efflux experiment, however, since the redistribution factor is largest at low cell density these experiments are less favourable. Efflux experiments would, however, be possible in a situation where the intracellular space has many field gradients caused by, for example, vesicles; see Fig. 2(*d*).

Transport equations

The equilibration of molecules in a cell suspension is described by the permeability equation:

$$\frac{dn_{out}}{dt} = -P(C_{out} - C_{in}) \quad (5)$$

where P is a permeability coefficient and C represents concentration. The solution of eqn. (5) for our conditions, where the total number of molecules, $n_{in}+n_{out}$, and the volume, $V_{in}+V_{out}$, are constant, is:

$$\Delta(t) = \Delta(0)\exp\left[-t\cdot P\left(\frac{1}{V_{in}}+\frac{1}{V_{out}}\right)\right] \qquad (6)$$

V_{in} and V_{out} can be related to the measurable parameter haematocrit. $H = V_{cells}/V_{total}$ when $V_{total} = V_{out}+V_{cell}$ and $V_{cell} = V_{in}+(1-b)V_{cell}$ because a fraction $1-b$ of the cells is already taken up by other molecules, e.g. haemoglobin. These relationships and eqn. (6) allow the parameter P to be determined from the observed half-life ($t_{\frac{1}{2}}$) in a plot of $\Delta(t)$ against time:

$$P = \frac{\ln 2 V_{in}(1-H)}{t_{\frac{1}{2}}[1+H(b-1)]} \qquad (7)$$

This value of P can then be used to give the transport rate in the usual units of mol/min per litre of cell water, where:

$$\text{Initial rate} = \frac{P\cdot C(0)_{out}}{V_{in}}$$

Results

Fig. 3 shows a typical sequence of proton n.m.r. spin-echo spectra obtained for L-alanine influx into human erythrocytes. The negative amplitude of the alanine methyl resonance is characteristic of a doublet in a spin-echo spectrum recorded with $2\tau =$ $1/J$ (see previous section) and increases with time as the alanine is transported from the magnetically inhomogeneous extracellular region into the intracellular region.

Two such time courses are plotted in Fig. 4 when it will be observed that the signal growth is greatly enhanced by the presence of small amounts of Dy–DTPA. Concentrations of 200–500 μM of this complex in the extracellular space could produce $\Delta(0)$ values of up to 0.95 depending on the haematocrit. At fixed cell concentrations and using the conditions described in Fig. 4, $\Delta(0)$ was found to be 0.38, 0.63, 0.85 and 0.90 for Dy–DTPA concentrations of 0, 0.3, 0.6 and 0.9 mM respectively.

Dy–DTPA does not appear to be transported across the cell membrane and, although profoundly affecting $\Delta(0)$, does not have any effect on the transport process for alanine as can be seen from the identical slopes of the two logarithmic plots shown in the inset of Fig. 4.

The practical application of the method for transport measurements as illustrated in Figs. 3 and 4 is quick and easy. A single 0.5 ml sample of cells is used for each complete run with routine sequential data acquisition from about 30 s until equilibrium. Fourier transformation of the series of accumulated decays acquired provides the peak intensities for the trans-

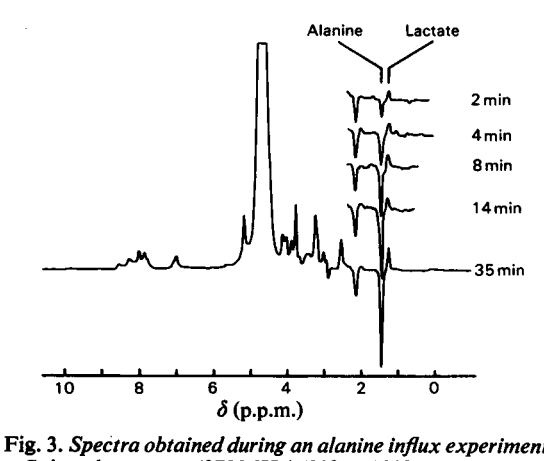

Fig. 3. *Spectra obtained during an alanine influx experiment*
Spin-echo spectra (270 MHz) (90°–τ–180°–τ sequence, $\tau = 60$ ms) of a suspension of human erythrocytes in ^2H_2O Krebs/Ringer medium. Other conditions were: sample volume, 0.5 ml; haematocrit, 80%; temperature 310K. At $t = 0$, 10 μl of 0.3 M-L-alanine was added to the sample. The signal at 1.4 p.p.m. is the unresolved doublet of alanine CH_3. Further assignments are given elsewhere (Brown *et al.*, 1977).

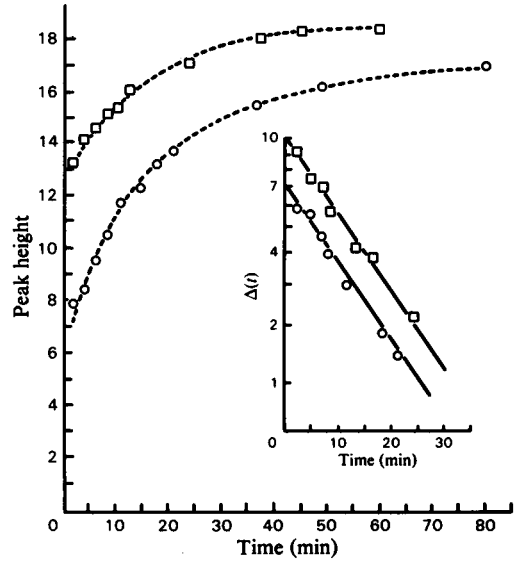

Fig. 4. *Plot of an alanine influx experiment*
The growth of the alanine CH_3 signal in influx experiments with L-alanine plotted as a function of time, □. Conditions as in Fig. 3; ○, 1 μl of 25 mM-Dy–DTPA added to the suspension before the influx experiment. Other conditions as for Fig. 3. Inset: logarithmic plots of $\Delta(t) = [R(\infty)-R(t)]/R(\infty)$ versus time.

port graphs directly. The peak intensities were usually normalized against the residual water resonance since this peak is the largest peak present and will reflect any small instrumental variations which occur. In H_2O samples, the water resonance is suppressed and any other strong peak, e.g. glutathione, may be used for normalization instead, although this is not always necessary (Brown *et al.*, 1977).

Measurement of alanine transport by other workers (e.g. Winter & Christensen, 1964; Young & Ellory, 1977) has revealed a marked stereospecificity between D- and L-alanine. The data in Fig. 5 confirm this point.

The data for lactate transport shown in Fig. 6 illustrate the method when applied to a much more rapidly transported species. The half-life for this process was 60s, giving a permeability parameter of 0.268 litre/min and an initial influx rate of 15mmol/min per litre for an external concentration of 60mM.

In the case of lactate, the metabolism of the transported molecule is significant and care must be taken in the interpretation of these results. Lactate is being produced continuously by the cell,

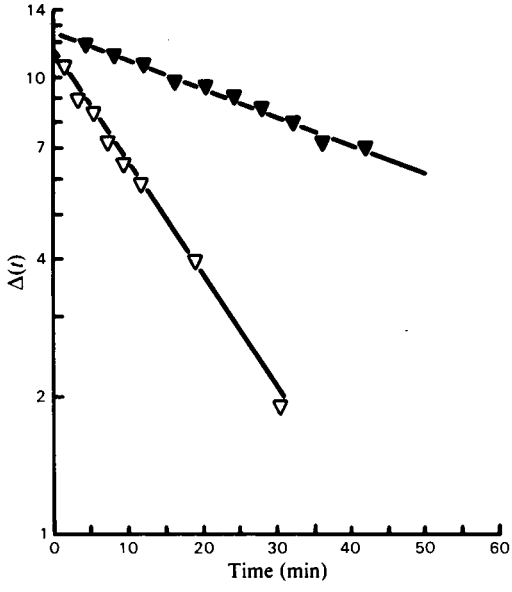

Fig. 5. *Comparison of* L-*alanine and* D-*alanine influx*
Uptake of L-alanine and D-alanine by human erythrocytes suspended in ^2H_2O Krebs/Ringer buffer. The logarithmic plots of $\Delta(t)$ are analogous to the inset in Fig. 4. At $t=0$ 10μl of a 0.3M-alanine solution and 2μl of a 25mM-Dy–DTPA solution were added to 0.5ml of cell suspension. Other conditions were: haematocrit, 83%; temperature, 310K. $t_{\frac{1}{2}}=10\pm0.5$min and 40 ± 2min for L-alanine and D-alanine respectively.

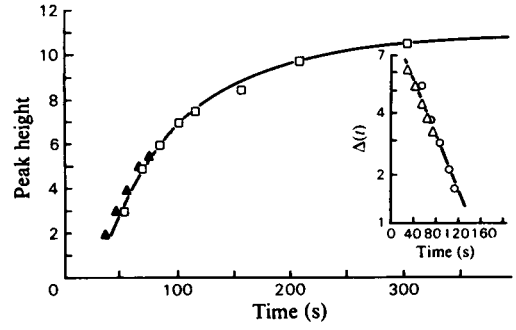

Fig. 6. L-*Lactate influx*
L-Lactate influx into human erythrocytes suspended in ^2H_2O Krebs/Ringer buffer. Plot of lactate CH_3 peak height, R versus time. At $t=0$ 15μl of 0.3M-lactate and 2μl of 25mM-Dy–DTPA were added to 0.5ml of cell suspension. Other conditions were: haematocrit, 85%; temperature, 310K. Inset: logarithmic plot analogous to inset in Fig. 4. $t_{\frac{1}{2}}=60\pm3$s.

but this rate is slow compared with the time scale of this experiment (see, for example, the lactate peaks at 1.3p.p.m. in Fig. 3). In ^2H_2O, we have shown that there is isotope exchange of lactate with solvent, both at the C-2 and C-3 positions (Brown *et al.*, 1977). The exchange at C-3 is very slow compared with rate of transport, but exchange at C-2 is relatively rapid. To eliminate any effect of exchange at C-2 on the observed C-3 methyl resonance, the C-2 resonance was irradiated, thus decoupling the two resonances, during the collection of the data shown in Fig. 6.

The magnetic susceptibility of the erythrocyte interior is readily controlled through the state of the haemoglobin. Experiments were performed in which erythrocytes were equilibrated with N_2/CO_2 (19:1) or treated with $NaNO_2$ to produce either deoxy- or met-haemoglobin. In oxy-haemoglobin, the iron is present as low-spin Fe^{2+}, which is diamagnetic, whereas in the deoxy- or met- forms, the haemoglobin contains high-spin Fe^{2+} or Fe^{3+}, both of which are paramagnetic. Under these conditions $\Delta(0)$ was always greater than 0, even in the absence of a paramagnetic complex.

It was in fact observed that the $\Delta(0)$ value for oxygenated erythrocytes varied widely from subject to subject (between 0 and 0.3 for 80% haematocrit). This could have been due to slight variations in cell shape as this would affect the amount of intracellular field gradients produced as shown in Fig. 2(*c*). It did not appear to be due to variations in the met- or deoxy-haemoglobin content of the cells since small increases in these constituents failed to reproduce the effects observed. It was also found that variations in transport rates for alanine of up to $\pm50\%$ were observed in erythrocytes from different people. This was much greater than the experimental error and

larger than the differences observed between samples prepared in ^1H_2O and ^2H_2O. At present the origin of these variations is not clear.

It was possible deliberately to increase the intracellular field gradients to the point where they exceed the extracellular gradients, thus causing a situation analogous to that shown in Fig. 2(*d*). It was found that $\Delta(0)$ then became negative, as expected.

No significant departure from the haematocrit dependence expected from eqn. (7) was observed in measurements of L-alanine transport when H was varied between 0.6 and 0.96.

Discussion

In the above experiments, n.m.r. has been used to provide information on the transport properties of cell suspensions. As a means of studying transport, it is ideal for influx into high-density cell suspensions or efflux from cells with high intracellular field inhomogeneity in low-cell-density samples. In cases where labelled analogues of the compounds under study are available, equilibrium transport could be studied as well.

The advantages of this method are that the measurements are quickly and easily taken and the results are very reproducible. The results given above are in agreement with the literature. The influx rate obtained for lactate in erythrocytes is comparable with that obtained by Halestrap (1976), and those for alanine agree with more recent values in the literature. The permeability for L- and D-alanine under the conditions used here were 1.1×10^{-2} litres/min and 2.8×10^{-3} litres/min respectively [assuming $b = 0.72$ (eqn. 7); Eilam & Stein (1974)]. The initial rates of influx under these conditions, where the extracellular alanine concentration was 35mM at $t = 0$, were 388 and 97.5μmol/min per 1 of cell water. Extrapolation to 0.2mM initial external concentration gives rates of 2.2 and 0.55μmol/min per 1 of cell water, which are in good agreement with the results of Young & Ellory (1977), who obtained 3.5 and 0.6mol/min per 1 of cell water. In making such an extrapolation it has been assumed that the K_m of the transporter for alanine exceeds 35mM. Extrapolation to 1mM initial external concentration gives an initial rate of 11 μmol/min per litre of cell water for L-alanine which is twice that obtained by Winter & Christensen (1964). On the basis of the relationship between amino acid concentration and uptake rate these workers proposed that there were two components of neutral amino acid uptake in human erythrocytes: a saturable system with a finite K_m value, and a non-saturable system. The presence of a non-saturable component, however, has been questioned by Young & Ellory (1977) and by Hoare (1972).

This method has a further advantage in that a great deal of metabolic information is obtainable concurrently, which may be of importance in assessing cell changes concomitant with the transport process.

This technique for measuring transport is not restricted to ^1H n.m.r. There are many compounds of interest that present proton spin-echo spectra that are difficult to observe, being highly coupled or masked by other resonances. However, these may often have ^{31}P or ^{13}C resonances which are much more accessible.

The method has several disadvantages. Owing to the inherent insensitivity of n.m.r., higher concentrations of compounds are required than may sometimes be desirable. As a result, under influx or efflux conditions ideal for n.m.r. measurement, the initial concentrations vary rapidly with time, which could make initial rates difficult to determine if non-exponential time courses occur. The limitations of sensitivity (about 1mM) and the greater difficulty of setting up equilibrium transport experiments make it somewhat less versatile than radioactive methods for obtaining basic kinetic parameters.

However, because of the dependence of $\Delta(0)$ on various cell parameters, this method of studying transport has several interesting implications which are not related to transport. Because $\Delta(0)$ depends strongly on cell shape and size, the time-dependence of such changes after a perturbation should be easily followed. Since $\Delta(0)$ also depends strongly on the diffusion term in eqn. (1), it is evident that qualitative information on the diffusion characteristics of molecules through the field gradients near the cell surface should be available. Finally, since the size and sign of $\Delta(0)$ depends on the relative field gradients inside and outside the cell, it may be possible to follow changes in the vesicle content of cells which generate vesicles of different magnetic susceptibility (e.g. phagocytosis) and to obtain information about molecular distribution within the different compartments of the cell.

The application of the method described in this paper should enable n.m.r. to provide a wide variety of useful information on whole cells in a relatively unperturbed state.

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