A p.m.r. isotope-exchange method for studying the kinetic properties of dehydrogenases in intact cells

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A method to determine the activity of dehydrogenases in an intact-cell system is described. The method involves the use of n.m.r. to monitor bulk isotope exchange. The approach is illustrated by application to the isotope equilibration of pyruvate and lactate as catalysed by lactate dehydrogenase in intact erythrocytes. Particular problems peculiar to bulk isotope exchange and its observation by n.m.r. are considered.

There is growing interest in non-invasive methods that allow the properties of enzymes in intact cells to be studied (see, e.g., Sies, 1980). These investigations may increase our understanding of the effect of the intracellular environment on enzymes, thus enabling more precise extrapolation of enzyme kinetic data obtained *in vitro* to be made. One approach is to develop methods that allow the comparison of enzyme properties *in situ* with those *in vitro*. Mechanistic information is not the primary goal of such studies.

Isotope-exchange techniques are a useful tool for these experiments. Spin-echo p.m.r. allows the labelling state of metabolites in suspensions of intact cells to be observed directly (Brindle *et al.*, 1980; Simpson *et al.*, 1981). The combination of the two methods has considerable potential advantages over the extraction and separation procedures used in radioisotope studies.

The strategy is to monitor the kinetics of label redistribution after 'pulsing' of the cells with label and to compare these results with an experiment performed *in vitro*. Certain criteria must be fulfilled before the kinetics observed *in situ* can be interpreted in terms of the rate of a single enzyme. (1) The pathway of label must be represented by a relatively simple mathematical model. This pathway may involve membrane transport, side reactions and net changes in substrate concentrations as well as the reaction of interest. This model will also be required to account for any perturbation introduced by the label itself, for example kinetic isotope effects. (2) There must be a direct relationship between the fraction of labelling and the n.m.r. signals.

The approach necessary to fulfil these criteria is illustrated in the present paper by the study of lactate dehydrogenase (EC 1.1.1.27) in intact human erythrocytes. Silverstein & Boyer (1964) demon-

strated that this enzyme can be studied by observing the equilibration of labelled methyl groups of lactate and pyruvate. In the present work the equilibration of 10 mM-L-[U-¹H]lactate and [²H₃]-pyruvate added to erythrocytes is observed by spin-echo p.m.r. The results of a detailed study of lactate dehydrogenase in the intact cell are presented and discussed in the following paper (Simpson *et al.*, 1982).

Experimental

Lactate dehydrogenase

Purified lactate dehydrogenase from human erythrocytes was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) as a suspension in (NH₄)₂SO₄ solution and generally used as such. Isotope-equilibration experiments were performed in 0.1 M-imidazole/²HCl buffer, pH* 7.4, in ²H₂O in the presence of appropriate quantities of enzyme, NAD⁺, [²H₃]pyruvate and [U-¹H]lactate. The final concentration of (NH₄)₂SO₄ in the equilibration experiments was typically about 20 mM. At high pyruvate concentrations, 400 mM-Na₂SO₄ has virtually no effect on pig H₄ lactate dehydrogenase (Rivedal & Sanner, 1979). Borgmann *et al.* (1974) also observed no effect of (NH₄)₂SO₄ on bovine heart lactate dehydrogenase at 5-fold higher concentrations than are used in the present work. The pH* (uncorrected pH-meter reading) was determined at the end of each experiment by using a pH electrode. Spectrophotometric lactate dehydrogenase assays were performed at 340 nm on portions of the samples at the end of the experiment. The assay mixture consisted of 0.1 M-imidazole/HCl buffer, pH 7.4, containing 0.1 mg of NADH/ml and 1 mM-pyruvate. Sigma pre-weighed NADH vials were used, as these give reproducible enzymic rates. This

makes it possible to compare determinations of lactate dehydrogenase activities made on different days (Howell, 1980). The mixture plus enzyme was preincubated for several minutes before the addition of pyruvate in order to dissociate any enzyme·pyruvate-NAD complex left over from the equilibration experiment.

Preparation of [$^2\text{H}_3$]pyruvate

Concentrated solutions of [$^2\text{H}_3$]pyruvate were prepared by incubating a mixture of crystalline horse myoglobin (200mg) (Sigma), 200mg of sodium pyruvate and 1 ml of $^2\text{H}_2\text{O}$ at 37°C for 6 h (the pH^* of the mixture was 7.5). The mixture was then diluted with 2 ml of $^2\text{H}_2\text{O}$, heated at 95°C for 5 min and centrifuged in an Eppendorf centrifuge (type 5142). The supernatant was decolorized with neutralized activated charcoal and freeze-dried. The solid was usually approx. 75% [$^2\text{H}_3$]pyruvate, the remainder being pyruvate dimer and higher polymers. Further purification (Von Korff, 1964) was performed on some batches by applying the supernatant to a column (15 cm $\times$ 1.5 cm) of Dowex 8 (Cl^- form) that had been equilibrated with deionized water. The pyruvate was eluted with a linear gradient of HCl produced by mixing deionized water and 0.1 M-HCl. The fractions containing pyruvate were identified by measuring the absorbance at 330 nm, pooled, neutralized with NaOH and freeze-dried. The pyruvate content was determined by spectrophotometric assay (Simpson *et al.*, 1981) and the deuteration (determined by p.m.r.) was greater than 95%. No difference was found between purified and non-purified preparations when tested in isotope-equilibration experiments or lactate dehydrogenase assays. The purified pyruvate usually contained about 1.5–2 mol of NaCl/mol of sodium pyruvate.

Preparation of [$3\text{-}^2\text{H}_3$]lactate

[$3\text{-}^2\text{H}_3$]Lactate was prepared by incubating a mixture of 200 mg of myoglobin, 200 mg of lithium lactate, 1 ml of $^2\text{H}_2\text{O}$, 200 units of bovine heart lactate dehydrogenase, 100 nmol of NAD^+ and 20 μmol of sodium pyruvate, $\text{pH}^* 7.5$, for 80 h at 37°C . The mixture was then heated and centrifuged as above and the supernatant applied to a column (15 cm $\times$ 1.5 cm) of Dowex 8 (Cl^- form). The column was washed with deionized water, followed by 1 M-HCl. The lactate was then eluted with 10 mM-HCl and neutralized with ZnCO_3 before being freeze-dried. The zinc salt was converted into the lithium salt by passage down a column of Dowex 50 (Li^+ form). The resulting lithium lactate (assayed with a Sigma assay kit) was approx. 65% deuterated at the C-3 position (determined from a comparison of the areas of the C-2 and C-3 proton resonances in the normal Fourier-transform spectrum). This

lactate was tested in a lactate dehydrogenase assay system containing 0.1 M-imidazole, $\text{pH} 7.4$, 10 mM-semicarbazide and 1 mM- NAD^+ by following the initial rate of NADH production spectrophotometrically at 340 nm. The initial velocity (v_0) was determined by fitting the curve obtained over the first 0.1 absorbance unit to a quadratic equation (a Nicolet 1180 computer was used for this) and taking the first derivative at $t = 0$.

P.m.r. methods

In general, spin-echo spectra were obtained as described previously (Brown *et al.*, 1977). In spin-echo spectra, the resonances of coupled protons show a phase modulation such that the echo is inverted when the delay between the 90° and 180° pulses (τ) equals $1/2J$. Coupling changes may be monitored very sensitively by observing at such a τ value (Brindle *et al.*, 1980). In order to monitor the methyl protons of lactate without interference from the C-2 proton, the effects of modulation must be minimized. This may be done by selecting τ to give an upright methyl resonance (for example 0–10 ms or 130–140 ms) or by applying a decoupling irradiation at the C-2 proton frequency during 2τ (Campbell & Dobson, 1979). In the experiments described below, τ and decoupling were selected as appropriate. In experiments with high-haematocrit erythrocytes (greater than 20%) at low τ values (less than 30 ms), irradiation was applied at the water frequency to minimize the haemoglobin background resonances by cross-relaxation (Rabenstein *et al.*, 1980).

Spectra either were processed with 20 Hz line broadening or a Lorentzian interpolation routine was employed to minimize digitization error in the determination of peak heights. Peak decays were analysed by using a Computer Technology CTL Mod 1 computer with a least-squares curve-fitting program based on the model described below. T_1 values were determined by using the inversion recovery method. Measurements in erythrocyte lysates were made by using a $(180^\circ - \Delta - 90^\circ - \tau - 180^\circ)_n$ pulse sequence with $\tau = 68$ ms (Campbell & Dobson, 1979).

Materials and general methods

The 100% NAD^+ was obtained from Boehringer Corp. (Lewes, East Sussex, U.K.). Other materials were obtained as described previously (Simpson *et al.*, 1981). The source and methods of preparing erythrocytes were as described previously (Simpson *et al.*, 1981), as were haematocrit determinations and haemoglobin and pyruvate assays. Glucose-depleted erythrocytes were obtained by incubating cells at low haematocrit (less than 10%) in $^1\text{H}_2\text{O}$ Krebs-Ringer buffer (Krebs & Henseleit, 1932) at 37°C for 1 h, sedimenting the cells, resuspending

them in fresh buffer and repeating the incubation before washing the cells into Krebs–Ringer buffer made with $^2\text{H}_2\text{O}$. Erythrocyte lysates were prepared by repeatedly freezing packed cell samples, contained in closed Eppendorf 1.5 ml micro test tubes (type 3810), in liquid N_2 and thawing at 37°C . Samples were stored at 4°C before use and warmed at 37°C for 5 min immediately before the isotope-exchange experiments.

In experiments involving cells, concentration refers to the free solution concentration. This was calculated by assuming a solution volume of 0.72 ml/ml of cells (Eilam & Stein, 1974). All experiments and assays were performed at 37°C .

Theory

Modelling of the exchange reaction

One may envisage a general system of metabolites capable of exchanging isotopic label:

$$A_1 = A_2 = \dots A_n$$

The first step may be membrane transport and the others may be enzymic or non-enzymic. The rate of any one step may be of particular interest. This means that, where possible, conditions should be manipulated to maximize the sensitivity of the exchange to the step of interest. Furthermore, rates of other steps that affect the overall exchange time course should be known.

At the concentrations used in the present study, the rate of transport of lactate and pyruvate into the cells is sufficiently rapid to be ignored in the kinetic treatment given below (Halestrap, 1976; Deuticke *et al.*, 1978; Brindle *et al.*, 1979). The lactate methyl hydrogen isotope-exchange system in human erythrocytes may be expressed thus (Brindle *et al.*, 1980):

A simpler description of the system can be obtained by making the following assumptions. (1) $k_{32} = 0$. The greater than 1000-fold difference in concentration between $^2\text{H}_2\text{O}$ and lactate plus pyruvate ensures that there is essentially no back flux. (2) $k_{12} = k_{21}$. There is a large difference between the concentration of added lactate and pyruvate (10 mM) and that of the nicotinamide dinucleotides (less than $200\text{ }\mu\text{M}$ total; Hasart *et al.*, 1972), and hence there is

no significant change in the former pair of substrates of lactate dehydrogenase in going to equilibrium. With these two assumptions, eqn. (1) simplifies to:

We may define A_1 as the concentration of $[\text{U-}^1\text{H}]\text{lactate}$, A_2 as the concentration of $[\text{H}_3]\text{-pyruvate}$, A_3 as the concentration of H_2O , A_i^* as the concentration of the deuterated forms and $A_i^0 = A_i + A_i^*$.

The use of such an equation can be justified if equilibrium between the compounds is achieved rapidly after the addition of the labelled species and if the system remains at equilibrium during the isotope-exchange time course. One may calculate that, when 10 mM-lactate and 10 mM-pyruvate are added to washed erythrocytes, about 1 nmol of NAD^+ and of lactate/ml are synthesized (Williamson *et al.*, 1967; Marshall & Omachi, 1974). In spite of the inhibition of human erythrocyte lactate dehydrogenase by 10 mM-pyruvate (Everse & Kaplan, 1973, 1975), there is still sufficient activity of the enzyme in human erythrocytes to establish an equilibrium in only a few seconds (Friedemann & Rapoport, 1974). As noted above, human erythrocyte lactate dehydrogenase is inhibited by the pyruvate concentration used in these experiments. The time required to establish this inhibition may, however, be calculated (Trommer *et al.*, 1979) to be less than 1 min after the addition of 10 mM-pyruvate to the sample. Similar arguments may be applied to the experiments with purified enzyme to show that equilibrium is achieved rapidly compared with the isotope-exchange time course. In the experiments with cells equilibrium is maintained by preventing

production of lactate and pyruvate, and thus the concentrations of these metabolites are constant during the experiment.

When isotope exchange is monitored by p.m.r., only bulk changes in isotopic labelling of a group can be observed, and if there is any significant isotope effect it cannot be ignored. In the present work the labels used are the proton and the deuteron, and one would expect a relatively small

Fig. 1. Effect of deuterium at the methyl position of pyruvate on the velocity of the reaction catalysed by human erythrocyte lactate dehydrogenase at 10 mM-pyruvate

Imidazole buffer (0.1 M) made with 90% $^2\text{H}_2\text{O}$ (pH* 7.4) containing $168 \mu\text{M}$ -NADH was preincubated at 37°C for 10 min with the appropriate quantities of $[\text{H}_3]$ pyruvate and $[\text{H}_3]$ pyruvate to yield a final concentration of 10 mM-pyruvate. A standard quantity of human erythrocyte lactate dehydrogenase was added and the reaction velocity (v) was determined from the decrease in absorbance at 340 nm. The results are presented normalized to the rate obtained with 10 mM- $[\text{H}_3]$ pyruvate (v_{H}). The line was calculated from eqn. (3) in the text with $K_{11} = 1.8 \text{ mM}$ and $K_{12} = 5 \text{ mM}$.

β -secondary isotope effect on the rate of the lactate dehydrogenase-catalysed reaction due to the replacement of the methyl protons of lactate or pyruvate by deuterons (Cook *et al.*, 1981). There is, in fact, a greater than 2-fold increase in the rate of reduction of 10 mM-pyruvate by NADH catalysed by human erythrocyte lactate dehydrogenase when the methyl group is deuterated (see Fig. 1). At lower pyruvate concentrations the effect is less; the rate is 1.4 ± 0.1 (S.D.) times as fast with 1 mM- $[\text{H}_3]$ pyruvate as with 1 mM- $[\text{H}_3]$ pyruvate, and lactate with 65%-deuterated methyl group yields a 1.2-fold lower initial rate of oxidation than does $[\text{U}-\text{H}]$ lactate. This strongly suggests that the large effect observed with 10 mM-pyruvate is an isotope effect on the inhibition process whereby high pyruvate concentrations inhibit H-type lactate dehydrogenase (Everse & Kaplan, 1973). This process has been postulated to proceed via formation of a pyruvate-NAD adduct at the methyl position of pyruvate; hence one would

expect a significant primary effect on the formation of the adduct due to the deuteration of the methyl group.

The dependence of the rate of the lactate dehydrogenase-catalysed reaction on the percentage deuteration of the methyl group at high pyruvate concentration may be modelled approximately by assuming two inhibition constants K_{11} and K_{12} for $[\text{H}_3]$ - and $[\text{H}_3]$ -pyruvate respectively. At a given concentration of pyruvate ($^1P + ^2P$) mM:

$$v = \frac{V_{\text{max.}}}{1 + \frac{^1P}{K_{11}} + \frac{^2P}{K_{12}}} \quad (2)$$

Solving for two values of ($^1P + ^2P$) mM (for example 1 mM and 10 mM) will yield values for K_{11} and K_{12} .

For the purposes of the present paper this semi-empirical model describes the isotope-dependence of the rate at 10 mM-pyruvate quite satisfactorily (Fig. 1). If it is assumed that eqn. (2) also describes the dependence of the equilibrium exchange velocity on the deuteration state of the pyruvate methyl group at 10 mM-pyruvate, then it may be used in conjunction with isotope-exchange equations to yield a model of the exchange kinetics of systems involving lactate dehydrogenase.

$$\begin{aligned} \frac{dA_1}{dt} &= k_{12} \cdot \frac{1}{1 + A_2/K_{11} + A_2^*/K_{12}} \cdot (A_2 - A_1) \\ \frac{dA_2}{dt} &= k_{12} \cdot \frac{1}{1 + A_2/K_{11} + A_2^*/K_{12}} \cdot (A_1 - A_2) - k_{23}A_2 \end{aligned} \quad (3)$$

The effect of deuteration of the lactate methyl group is assumed to be small enough to ignore (see above).

The solutions to these differential equations for particular initial conditions, k_{ij} and K_i values were obtained numerically by using the fourth-order Runge and Kutta method (see McCalla, 1967). Solutions of eqn. (3) for a particular initial condition are families of curves (such as those shown in Fig. 3). The initial conditions $A_1/A_1^0 = 1$, $A_2/A_2^0 = 1$ and $A_3/A_3^0 = 0$ correspond to the experiment of adding 10 mM- $[\text{H}_3]$ pyruvate and 10 mM- $[\text{U}-\text{H}]$ lactate to erythrocytes in $^2\text{H}_2\text{O}$ buffer. This experiment has been reported previously (Brindle *et al.*, 1980). Investigation of the solutions reveals that the initial conditions $A_1/A_1^0 = 1$, $A_2/A_2^0 = 0$ and $A_3/A_3^0 = 0$ yield time courses that allow more accurate determinations of k_{12} over a wide range of values. This is because high methyl-group equilibration rates can be determined by diluting the system and lowering the rate. It is also found that the value of k_{23} need only be known approximately, as the time courses are relatively insensitive to changes in this rate constant.

Results and discussion

Relationship of peak height and fractional labelling of chemical species

In the p.m.r. experiments described in the present paper, the height of the lactate methyl-group resonance in spin-echo spectra is used as a measure of the overall concentration of protons in lactate methyl groups in the sample. In practice, we may expect the formation of $[3\text{-}^1\text{H}_3]\text{-}$, $[3\text{-}^1\text{H}_2]\text{-}$ and $[3\text{-}^1\text{H}]\text{-}$ lactate. Each of these compounds has methyl-group proton resonances characterized by specific chemical shifts and relaxation times. In these experiments, large line broadening (usually more than 10 Hz) was applied during data processing in order to improve the signal-to-noise ratio and minimize digitization errors in peak height measurements (broad peaks are characterized by more data points within the computer memory than are narrow peaks for a given digital resolution). Another effect of broadening is to render the splitting of the resonance due to the formation of these chemically shifted partially labelled lactate species insignificant. The linewidths in whole-cell experiments were typically greater than 35 Hz, whereas the chemical-shift differences between these species were less than 12 Hz. It is possible to show that splittings of less than one-third of the linewidth have no effect on the peak height of broadened resonances (Simpson, 1981).

In order to collect data efficiently it was desirable to pulse at a rate much greater than $1/(5 \times T_1)$. In such a situation, changes in T_1 during isotope exchange at a multiply labelled site (for example the lactate methyl group) could distort the time course of peak intensity because of saturation. This effect is particularly noticeable with molecules in free solution, where T_1 values can be greater than 10 s. The T_1 values of the individual labelled lactate species $[3\text{-}^1\text{H}_3]\text{-}$, $[3\text{-}^1\text{H}_2]\text{-}$ and $[3\text{-}^1\text{H}]\text{-}$ lactate are 1.5 ± 0.1 s, 2.3 ± 0.1 s and 5.4 ± 0.1 s (means $\pm$ s.e.m.) respectively in packed erythrocyte lysates. In order to test whether this was significant, some experiments in cells were performed both at slow (7 scans/min) and fast (56 scans/min) repetition rates. Some time courses were also followed by accumulating alternate spectra at the different scan rates. In all cases, no significant effect of scan repetition rate was observed.

The problem of T_2 effects on the observation of isotope exchange was investigated in a previous paper (Simpson *et al.*, 1981). No effect on exchange was observed on varying the spin-echo delay τ .

Application of the model to human erythrocyte lactate dehydrogenase

The model described in the Theory section may be applied to studies of human erythrocyte lactate

Fig. 2. Methyl-group equilibration between 10 mM-[U- ^1H]lactate and 10 mM-[$^2\text{H}_3$]pyruvate catalysed by human erythrocyte lactate dehydrogenase

Conditions are as described in the legend for Table 1. The lactate dehydrogenase activity was 6.0 units ($\mu\text{mol/min}$). Enzyme, lactate, buffer and NAD^+ were incubated at 37°C for 5 min before the experiment. (a) Successive 1 min spectra (normal Fourier transform) accumulated after the addition, at $t = 0$, of the [$^2\text{H}_3$]pyruvate. The left-hand peak is pyruvate and the right-hand peak is lactate. (b) Lactate peak intensity, normalized to a buffer peak and the initial intensity, from the above spectra plotted against time. The line was obtained from the model described in the Theory section with $k_{23} = 0$, $k_{12} = 0.0756 \text{ min}^{-1}$, $K_{11} = 1.8 \text{ mM}$ and $K_{12} = 5 \text{ mM}$.

dehydrogenase. The concentrations of lactate and pyruvate chosen for all experiments were 10 mM because this concentration is rapidly transported into erythrocytes, is large compared with endogenous concentrations (less than 0.5 mM in glucose-depleted cells) and is easily observed by spin-echo p.m.r. These concentrations also yield exchange kinetics that are sensitive to lactate dehydrogenase activity.

Fig. 2(b) shows an example of the application of the model to the equilibration of 10 mM-[$^2\text{H}_3$]-

pyruvate with 10 mM-[U-¹H]lactate catalysed by purified human erythrocyte lactate dehydrogenase (k_{23} is negligible in such experiments). The line fit was obtained by least squares. Such experiments may be used to evaluate k_{12} , the methyl-group equilibration rate constant. It is shown in Table 1 that the fitted k_{12} value is linearly related to the activity of lactate dehydrogenase in the sample.

With intact erythrocytes, several different isotope-equilibration experiments are possible; however, the simplest comparison between the pure enzyme and the enzyme *in situ* is obtained by performing the analogous experiments in the whole cell, that is adding 10 mM-[²H₃]pyruvate and 10 mM-[U-¹H]lactate to cells in ²H₂O.

In order to investigate the adequacy of the model at describing exchange kinetics in whole cells, the dependence of the methyl-group equilibration rate on haematocrit was studied. These experiments require knowledge of an approximate value for k_{23} , the pyruvate/water exchange rate constant. The effect of lactate on this rate constant was investigated by using freeze-thaw-lysed human erythrocytes, which have no free NAD⁺ owing to the action of NAD⁺ glycohydrolase (EC 3.2.2.5) (Solti & Friedrich, 1979). As shown in Table 2, lactate has

no significant effect on k_{23} , which is linearly related to the haematocrit of the sample.

Time courses for the methyl-group equilibration experiment are shown in Fig. 3, with least-squares-fitted lines. The dependence of the measured k_{12} on haematocrit is shown in Fig. 4. The slight curvature at high haematocrits is caused by the loss of free solution volume to haemoglobin, which raises the effective lactate dehydrogenase concentration. In this experiment the quantities of [U-¹H]lactate and [²H₃]pyruvate added were adjusted to take account of this loss of solution volume (see the Experimental section). Another problem at high haematocrits is that the cells produce significant amounts of lactate from glucose, and pyruvate from 2,3-bisphosphoglycerate (Whittam, 1958). These problems were

Table 1. Specific methyl-group equilibration rates for human erythrocyte lactate dehydrogenase

These rates were determined in 0.5 ml of 0.1 M-imidazole buffer, pH* 7.45 ± 0.05, containing *v* units of lactate dehydrogenase (assayed as described in the Experimental section), 10 mM-[²H₃]pyruvate, 10 mM-[U-¹H]lactate and 100 μM-NAD⁺. The standard error is derived from the deviations of the experimental peak-height determinations from the lines fitted by using the model described in the text.

<i>v</i> (μmol/min)	10 ⁻³ × Specific methyl-group equilibration rate constant (k_{12}/v) (μmol ⁻¹)
9.5	12.9 ± 0.3
2.0	12.4 ± 0.2
6.0	12.6 ± 0.1

Fig. 3. Time courses of the lactate methyl-group resonance in spin-echo spectra of erythrocytes in ²H₂O Krebs-Ringer buffer after addition of 10 mM-[U-¹H]-lactate and 10 mM-[²H₃]pyruvate at *t* = 0

The haematocrit and hence both k_{12} and k_{23} are varied. The lines were obtained from the model described in the Theory section by varying k_{12} until the sum of the squares of the deviations of the experimental points from the line calculated by using the k_{12} values was minimized.

Table 2. Effect of dilution and lactate on the solvent exchange of pyruvate hydrogen atoms in erythrocytes and erythrocyte lysate

[¹H₃]Pyruvate (10 mM) was added to the sample (0.5 ml of erythrocytes in ²H₂O Krebs-Ringer buffer) at *t* = 0, and the exponential decay constant of the pyruvate peak in τ = 60 ms spin-echo spectra was determined (Simpson *et al.*, 1981). For the lactate experiment the sample was lysed by freezing with liquid N₂ and thawing at 37°C three times before the exchange measurement. Values are given as means ± S.E.M.

Treatment	10 ⁵ × Specific exchange constant (min ⁻¹ · ml ⁻¹ per mg of haemoglobin)
Control (81% haematocrit)	19.0 ± 2.0
4-fold-diluted cells in Krebs-Ringer buffer (20% haematocrit)	20.1 ± 2.0
Lysate + 10 mM-[U- ¹ H]lactate (81% initial haematocrit)	18.4 ± 2.0

Fig. 4. Dependence of k_{12} in intact erythrocytes on haematocrit

The fitted k_{12} values obtained from several time courses, such as those in Fig. 3, for cells from a single individual, are plotted against the haematocrit of the sample. The line was calculated by assuming that k_{12} was linearly related to the lactate dehydrogenase concentration of the sample. The slight curvature is due to the increase in concentration of the enzyme at high haematocrits caused by the significant loss of solution volume to haemoglobin. The lactate and pyruvate additions were corrected for this loss of solution volume.

avoided by using glucose-depleted cells (see the Experimental section) and by adding 5 mM-NaF (Whittam, 1958).

In conclusion, it is possible, by using isotope exchange, to extract a parameter, k_{12} , that is a measure of the activity of lactate dehydrogenase under specific conditions in whole erythrocytes. This parameter may also be obtained from experiments with purified human erythrocyte lactate dehydrogenase, and accurate comparisons can thus be made between the enzyme in the purified state and *in situ* in the intact erythrocyte. The results of such a comparison are presented in the following paper (Simpson *et al.*, 1982).

Several other experiments, analogous to the one discussed in detail in the present paper, may be envisaged. The equilibration of the substrates of malate dehydrogenase (EC 1.1.1.37) and β -hydroxybutyrate dehydrogenase (EC 1.1.1.30) are examples. Some of these may also be expected to show kinetics similar to those reported in the present paper.

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